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(54) Title: METHOD FOR THE PRODUCTION OF 1,3-PROPANEDIOL BY RECOMBINANT ORGANISMS

## (57) Abstract

Recombinant organisms are provided comprising genes encoding glycerol-3-phosphate dehydrogenase, glycerol-3-phosphatase, glycerol dehydratase and 1,3-propanediol oxidoreductase activities useful for the production of 1,3-propanediol from a variety of carbon substrates.

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TITLEMETHOD FOR THE PRODUCTION OF 1,3-PROPANEDIOL  
BY RECOMBINANT ORGANISMSFIELD OF INVENTION

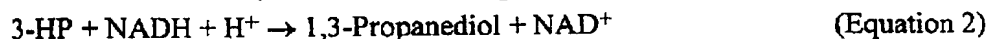
5       The present invention relates to the field of molecular biology and the use of recombinant organisms for the production of desired compounds. More specifically it describes the expression of cloned genes for glycerol-3-phosphate dehydrogenase (G3PDH) and glycerol-3-phosphatase (G3P phosphatase), glycerol dehydratase (*dhaB*), and 1,3-propanediol oxidoreductase (*dhaT*), either separately  
10       or together, for the enhanced production of 1,3-propanediol.

BACKGROUND

1,3-Propanediol is a monomer having potential utility in the production of polyester fibers and the manufacture of polyurethanes and cyclic compounds.

A variety of chemical routes to 1,3-propanediol are known. For example  
15       ethylene oxide may be converted to 1,3-propanediol over a catalyst in the presence of phosphine, water, carbon monoxide, hydrogen and an acid, by the catalytic solution phase hydration of acrolein followed by reduction, or from hydrocarbons such as glycerol, reacted in the presence of carbon monoxide and hydrogen over catalysts having atoms from group VIII of the periodic table. Although it is  
20       possible to generate 1,3-propanediol by these methods, they are expensive and generate waste streams containing environmental pollutants.

It has been known for over a century that 1,3-propanediol can be produced from the fermentation of glycerol. Bacterial strains able to produce 1,3-propanediol have been found, for example, in the groups *Citrobacter*, *Clostridium*,  
25       *Enterobacter*, *Ilyobacter*, *Klebsiella*, *Lactobacillus*, and *Pelobacter*. In each case studied, glycerol is converted to 1,3-propanediol in a two step, enzyme catalyzed reaction sequence. In the first step, a dehydratase catalyzes the conversion of glycerol to 3-hydroxypropionaldehyde (3-HP) and water (Equation 1). In the second step, 3-HP is reduced to 1,3-propanediol by a NAD<sup>+</sup>-linked  
30       oxidoreductase (Equation 2).



35       The 1,3-propanediol is not metabolized further and, as a result, accumulates in high concentration in the media. The overall reaction consumes a reducing equivalent in the form of a cofactor, reduced  $\beta$ -nicotinamide adenine dinucleotide (NADH), which is oxidized to nicotinamide adenine dinucleotide (NAD<sup>+</sup>).

The production of 1,3-propanediol from glycerol is generally performed under anaerobic conditions using glycerol as the sole carbon source and in the absence of other exogenous reducing equivalent acceptors. Under these conditions, in for example, strains of *Citrobacter*, *Clostridium*, and *Klebsiella*, a parallel pathway for glycerol operates which first involves oxidation of glycerol to dihydroxyacetone (DHA) by a NAD<sup>+</sup>- (or NADP<sup>+</sup>-) linked glycerol dehydrogenase (Equation 3). The DHA, following phosphorylation to dihydroxyacetone phosphate (DHAP) by a DHA kinase (Equation 4), becomes available for biosynthesis and for supporting ATP generation via, for example, glycolysis.



In contrast to the 1,3-propanediol pathway, this pathway may provide carbon and energy to the cell and produces rather than consumes NADH.

In *Klebsiella pneumoniae* and *Citrobacter freundii*, the genes encoding the functionally linked activities of glycerol dehydratase (*dhaB*), 1,3-propanediol oxidoreductase (*dhaT*), glycerol dehydrogenase (*dhaD*), and dihydroxyacetone kinase (*dhaK*) are encompassed by the *dha* regulon. The *dha* regulons from *Citrobacter* and *Klebsiella* have been expressed in *Escherichia coli* and have been shown to convert glycerol to 1,3-propanediol.

Biological processes for the preparation of glycerol are known. The overwhelming majority of glycerol producers are yeasts, but some bacteria, other fungi and algae are also known to produce glycerol. Both bacteria and yeasts produce glycerol by converting glucose or other carbohydrates through the fructose-1,6-bisphosphate pathway in glycolysis or by the Embden Meyerhof Parnas pathway, whereas, certain algae convert dissolved carbon dioxide or bicarbonate in the chloroplasts into the 3-carbon intermediates of the Calvin cycle. In a series of steps, the 3-carbon intermediate, phosphoglyceric acid, is converted to glyceraldehyde 3-phosphate which can be readily interconverted to its keto isomer dihydroxyacetone phosphate and ultimately to glycerol.

Specifically, the bacteria *Bacillus licheniformis* and *Lactobacillus lycopersica* synthesize glycerol, and glycerol production is found in the halotolerant algae *Dunaliella sp.* and *Asteromonas gracilis* for protection against high external salt concentrations (Ben-Amotz et al., *Experientia* 38, 49-52, (1982)). Similarly, various osmotolerant yeasts synthesize glycerol as a protective measure. Most strains of *Saccharomyces* produce some glycerol during alcoholic fermentation, and this can be increased physiologically by the application of

osmotic stress (Albertyn et al., *Mol. Cell. Biol.* 14, 4135-4144, (1994)). Earlier this century commercial glycerol production was achieved by the use of *Saccharomyces* cultures to which "steering reagents" were added such as sulfites or alkalis. Through the formation of an inactive complex, the steering agents  
5 block or inhibit the conversion of acetaldehyde to ethanol; thus, excess reducing equivalents (NADH) are available to or "steered" towards DHAP for reduction to produce glycerol. This method is limited by the partial inhibition of yeast growth that is due to the sulfites. This limitation can be partially overcome by the use of alkalis which create excess NADH equivalents by a different mechanism. In this  
10 practice, the alkalis initiated a Cannizzaro disproportionation to yield ethanol and acetic acid from two equivalents of acetaldehyde.

The gene encoding glycerol-3-phosphate dehydrogenase (DAR1, GPD1) has been cloned and sequenced from *S. diastaticus* (Wang et al., *J. Bact.* 176, 7091-7095, (1994)). The DAR1 gene was cloned into a shuttle vector and used to  
15 transform *E. coli* where expression produced active enzyme. Wang et al. (supra) recognize that DAR1 is regulated by the cellular osmotic environment but do not suggest how the gene might be used to enhance 1,3-propanediol production in a recombinant organism.

Other glycerol-3-phosphate dehydrogenase enzymes have been isolated:  
20 for example, sn-glycerol-3-phosphate dehydrogenase has been cloned and sequenced from *S. cerevisiae* (Larason et al., *Mol. Microbiol.* 10, 1101, (1993)) and Albertyn et al., (*Mol. Cell. Biol.* 14, 4135, (1994)) teach the cloning of GPD1 encoding a glycerol-3-phosphate dehydrogenase from *S. cerevisiae*. Like Wang et al. (supra), both Albertyn et al. and Larason et al. recognize the osmo-sensitivity  
25 of the regulation of this gene but do not suggest how the gene might be used in the production of 1,3-propanediol in a recombinant organism.

As with G3PDH, glycerol-3-phosphatase has been isolated from *Saccharomyces cerevisiae* and the protein identified as being encoded by the GPP1 and GPP2 genes (Norbeck et al., *J. Biol. Chem.* 271, 13875, (1996)). Like  
30 the genes encoding G3PDH, it appears that GPP2 is osmosensitive.

Although biological methods of both glycerol and 1,3-propanediol production are known, it has never been demonstrated that the entire process can be accomplished by a single recombinant organism.

Neither the chemical nor biological methods described above for the  
35 production of 1,3-propanediol are well suited for industrial scale production since the chemical processes are energy intensive and the biological processes require the expensive starting material, glycerol. A method requiring low energy input and an inexpensive starting material is needed. A more desirable process would

incorporate a microorganism that would have the ability to convert basic carbon sources such as carbohydrates or sugars to the desired 1,3-propanediol end-product.

Although a single organism conversion of fermentable carbon source other than glycerol or dihydroxyacetone to 1,3-propanediol would be desirable, it has been documented that there are significant difficulties to overcome in such an endeavor. For example, Gottschalk et al. (EP 373 230) teach that the growth of most strains useful for the production of 1,3-propanediol, including *Citrobacter freundii*, *Clostridium autobutylicum*, *Clostridium butylicum*, and *Klebsiella pneumoniae*, is disturbed by the presence of a hydrogen donor such as fructose or glucose. Strains of *Lactobacillus brevis* and *Lactobacillus buchneri*, which produce 1,3-propanediol in co-fermentations of glycerol and fructose or glucose, do not grow when glycerol is provided as the sole carbon source, and, although it has been shown that resting cells can metabolize glucose or fructose, they do not produce 1,3-propanediol. (Veiga DA Cunha et al., *J. Bacteriol.* 174, 1013 (1992)). Similarly, it has been shown that a strain of *Ilyobacter polytropus*, which produces 1,3-propanediol when glycerol and acetate are provided, will not produce 1,3-propanediol from carbon substrates other than glycerol, including fructose and glucose. (Steib et al., *Arch. Microbiol.* 140, 139 (1984)). Finally Tong et al. (*Appl. Biochem. Biotech.* 34, 149 (1992)) has taught that recombinant *Escherichia coli* transformed with the *dha* regulon encoding glycerol dehydratase does not produce 1,3-propanediol from either glucose or xylose in the absence of exogenous glycerol.

Attempts to improve the yield of 1,3-propanediol from glycerol have been reported where co-substrates capable of providing reducing equivalents, typically fermentable sugars, are included in the process. Improvements in yield have been claimed for resting cells of *Citrobacter freundii* and *Klebsiella pneumoniae* DSM 4270 cofermenting glycerol and glucose (Gottschalk et al., *supra.*, and Tran-Dinh et al., DE 3734 764); but not for growing cells of *Klebsiella pneumoniae* ATCC 25955 cofermenting glycerol and glucose, which produced no 1,3-propanediol (I-T. Tong, Ph.D. Thesis, University of Wisconsin-Madison (1992)). Increased yields have been reported for the cofermentation of glycerol and glucose or fructose by a recombinant *Escherichia coli*; however, no 1,3-propanediol is produced in the absence of glycerol (Tong et al., *supra.*). In these systems, single organisms use the carbohydrate as a source of generating NADH while providing energy and carbon for cell maintenance or growth. These disclosures suggest that sugars do not enter the carbon stream that produces 1,3-propanediol. In no case is 1,3-propanediol produced in the absence of an

exogenous source of glycerol. Thus the weight of literature clearly suggests that the production of 1,3-propanediol from a carbohydrate source by a single organism is not possible.

5 The problem to be solved by the present invention is the biological production of 1,3-propanediol by a single recombinant organism from an inexpensive carbon substrate such as glucose or other sugars. The biological production of 1,3-propanediol requires glycerol as a substrate for a two step sequential reaction in which a dehydratase enzyme (typically a coenzyme B<sub>12</sub>-dependent dehydratase) converts glycerol to an intermediate, 3-hydroxy-propionaldehyde, which is then reduced to 1,3-propanediol by a NADH- (or  
10 NADPH) dependent oxidoreductase. The complexity of the cofactor requirements necessitates the use of a whole cell catalyst for an industrial process which utilizes this reaction sequence for the production of 1,3-propanediol. Furthermore, in order to make the process economically viable, a less expensive feedstock than  
15 glycerol or dihydroxyacetone is needed. Glucose and other carbohydrates are suitable substrates, but, as discussed above, are known to interfere with 1,3-propanediol production. As a result no single organism has been shown to convert glucose to 1,3-propanediol.

Applicants have solved the stated problem and the present invention  
20 provides for bioconverting a fermentable carbon source directly to 1,3-propanediol using a single recombinant organism. Glucose is used as a model substrate and the bioconversion is applicable to any existing microorganism. Microorganisms harboring the genes encoding glycerol-3-phosphate dehydrogenase (G3PDH), glycerol-3-phosphatase (G3P phosphatase), glycerol  
25 dehydratase (*dhaB*), and 1,3-propanediol oxidoreductase (*dhaT*), are able to convert glucose and other sugars through the glycerol degradation pathway to 1,3-propanediol with good yields and selectivities. Furthermore, the present invention may be generally applied to include any carbon substrate that is readily converted to 1) glycerol, 2) dihydroxyacetone, or 3) C<sub>3</sub> compounds at the  
30 oxidation state of glycerol (e.g., glycerol 3-phosphate) or 4) C<sub>3</sub> compounds at the oxidation state of dihydroxyacetone (e.g., dihydroxyacetone phosphate or glyceraldehyde 3-phosphate).

#### SUMMARY OF THE INVENTION

The present invention provides a method for the production of  
35 1,3-propanediol from a recombinant organism comprising:

- (i) transforming a suitable host organism with a transformation cassette comprising at least one of (a) a gene encoding a glycerol-3-phosphate dehydrogenase activity; (b) a gene encoding a glycerol-3 phosphatase activity;

(c) genes encoding a dehydratase activity; and (d) a gene encoding 1,3-propanediol oxidoreductase activity, provided that if the transformation cassette comprises less than all the genes of (a)-(d), then the suitable host organism comprises endogenous genes whereby the resulting transformed host organism comprises at least one of each of genes (a)-(d);

(ii) culturing the transformed host organism under suitable conditions in the presence of at least one carbon source selected from the group consisting of monosaccharides, oligosaccharides, polysaccharides, or a one carbon substrate whereby 1,3-propanediol is produced; and

(iii) recovering the 1,3-propanediol.

The invention further provides transformed hosts comprising expression cassettes capable of expressing glycerol-3-phosphate dehydrogenase, glycerol-3-phosphatase, glycerol dehydratase and 1,3-propanediol oxidoreductase activities for the production of 1,3-propanediol.

The suitable host organism used in the method is selected from the group consisting of bacteria, yeast, and filamentous fungi. The suitable host organism is more particularly selected from the group of genera consisting of *Citrobacter*, *Enterobacter*, *Clostridium*, *Klebsiella*, *Aerobacter*, *Lactobacillus*, *Aspergillus*, *Saccharomyces*, *Schizosaccharomyces*, *Zygosaccharomyces*, *Pichia*, *Kluyveromyces*, *Candida*, *Hansenula*, *Debaryomyces*, *Mucor*, *Torulopsis*, *Methylobacter*, *Escherichia*, *Salmonella*, *Bacillus*, *Streptomyces* and *Pseudomonas*. Most particularly, the suitable host organism is selected from the group consisting of *E. coli*, *Klebsiella spp.*, and *Saccharomyces spp.* Particular transformed host organisms used in the method are 1) a *Saccharomyces spp.* transformed with a transformation cassette comprising the genes *dhaB1*, *dhaB2*, *dhaB3*, and *dhaT*, wherein the genes are stably integrated into the *Saccharomyces spp.* genome; and 2) a *Klebsiella spp.* transformed with a transformation cassette comprising the genes GPD1 and GPD2;

The preferred carbon source of the invention is glucose.

The method further uses the gene encoding a glycerol-3-phosphate dehydrogenase enzyme selected from the group consisting of genes corresponding to amino acid sequences given in SEQ ID NO:11, in SEQ ID NO:12, and in SEQ ID NO:13, the amino acid sequences encompassing amino acid substitutions, deletions or additions that do not alter the function of the glycerol-3-phosphate dehydrogenase enzyme. The method also uses the gene encoding a glycerol-3-phosphatase enzyme selected from the group consisting of genes corresponding to amino acid sequences given in SEQ ID NO:33 and in SEQ ID NO:17, the amino acid sequences encompassing amino acid substitutions, deletions or additions that



do not alter the function of the glycerol-3-phosphatase enzyme. The method also uses the gene encoding a glycerol kinase enzyme that corresponds to an amino acid sequence given in SEQ ID NO:18, the amino acid sequence encompassing amino acid substitutions, deletions or additions that do not alter the function of the glycerol kinase enzyme. The method also uses the genes encoding a dehydratase enzyme comprise dhaB1, dhaB2 and dhB3, the genes corresponding respectively to amino acid sequences given in SEQ ID NO:34, SEQ ID NO:35, and SEQ ID NO:36, the amino acid sequences encompassing amino acid substitutions, deletions or additions that do not alter the function of the dehydratase enzyme.

The method also uses the gene encoding a 1,3-propanediol oxidoreductase enzyme that corresponds to an amino acid sequence given in SEQ ID NO:37, the amino acid sequence encompassing amino acid substitutions, deletions or additions that do not alter the function of the 1,3-propanediol oxidoreductase enzyme.

The invention is also embodied in a transformed host cell comprising:

- (a) a group of genes comprising
  - (1) a gene encoding a glycerol-3-phosphate dehydrogenase enzyme corresponding to the amino acid sequence given in SEQ ID NO:11;
  - (2) a gene encoding a glycerol-3-phosphatase enzyme corresponding to the amino acid sequence given in SEQ ID NO:17;
  - (3) a gene encoding the  $\alpha$  subunit of the glycerol dehydratase enzyme corresponding to the amino acid sequence given in SEQ ID NO:34;
  - (4) a gene encoding the  $\beta$  subunit of the glycerol dehydratase enzyme corresponding to the amino acid sequence given in SEQ ID NO:35;
  - (5) a gene encoding the  $\gamma$  subunit of the glycerol dehydratase enzyme corresponding to the amino acid sequence given in SEQ ID NO:36; and
  - (6) a gene encoding the 1,3-propanediol oxidoreductase enzyme corresponding to the amino acid sequence given in SEQ ID NO:37,
 the respective amino acid sequences of (a)(1)-(6) encompassing amino acid substitutions, deletions, or additions that do not alter the function of the enzymes of genes (1)-(6), and
- (b) a host cell transformed with the group of genes of (a), whereby the transformed host cell produces 1,3-propanediol on at least one substrate selected from the group consisting of monosaccharides, oligosaccharides, and polysaccharides or from a one-carbon substrate.

#### BRIEF DESCRIPTION OF BIOLOGICAL DEPOSITS AND SEQUENCE LISTING

The transformed *E. coli* W2042 (comprising the *E. coli* host W1485 and plasmids pDT20 and pAH42) containing the genes encoding glycerol-3-phosphate

dehydrogenase (G3PDH) and glycerol-3-phosphatase (G3P phosphatase), glycerol dehydratase (*dhaB*), and 1,3-propanediol oxidoreductase (*dhaT*) was deposited on 26 September 1996 with the ATCC under the terms of the Budapest Treaty on the International Recognition of the Deposit of Micro-organisms for the Purpose of Patent Procedure and is designated as ATCC 98188.

*S. cerevisiae* YPH500 harboring plasmids pMCK10, pMCK17, pMCK30 and pMCK35 containing genes encoding glycerol-3-phosphate dehydrogenase (G3PDH) and glycerol-3-phosphatase (G3P phosphatase), glycerol dehydratase (*dhaB*), and 1,3-propanediol oxidoreductase (*dhaT*) was deposited on 26 September 1996 with the ATCC under the terms of the Budapest Treaty on the International Recognition of the Deposit of Micro-organisms for the Purpose of Patent Procedure and is designated as ATCC 74392.

"ATCC" refers to the American Type Culture Collection international depository located at 12301 Parklawn Drive, Rockville, MD 20852 U.S.A. The designations refer to the accession number of the deposited material.

Applicants have provided 49 sequences in conformity with Rules for the Standard Representation of Nucleotide and Amino Acid Sequences in Patent Applications (Annexes I and II to the Decision of the President of the EPO, published in Supplement No. 2 to OJ EPO, 12/1992) and with 37 C.F.R. 1.821-1.825 and Appendices A and B (Requirements for Application Disclosures Containing Nucleotides and/or Amino Acid Sequences).

#### DETAILED DESCRIPTION OF THE INVENTION

The present invention provides a method for a biological production of 1,3-propanediol from a fermentable carbon source in a single recombinant organism. The method incorporates a microorganism containing genes encoding glycerol-3-phosphate dehydrogenase (G3PDH), glycerol-3-phosphatase (G3P phosphatase), glycerol dehydratase (*dhaB*), and 1,3-propanediol oxidoreductase (*dhaT*). The recombinant microorganism is contacted with a carbon substrate and 1,3-propanediol is isolated from the growth media.

The present method provides a rapid, inexpensive and environmentally responsible source of 1,3-propanediol monomer useful in the production of polyesters and other polymers.

The following definitions are to be used to interpret the claims and specification.

The terms "glycerol dehydratase" or "dehydratase enzyme" refer to the polypeptide(s) responsible for an enzyme activity that is capable of isomerizing or converting a glycerol molecule to the product 3-hydroxypropionaldehyde. For the purposes of the present invention the dehydratase enzymes include a glycerol

dehydratase (GenBank U09771, U30903) and a diol dehydratase (GenBank D45071) having preferred substrates of glycerol and 1,2-propanediol, respectively. Glycerol dehydratase of *K. pneumoniae* ATCC 25955 is encoded by the genes *dhaB1*, *dhaB2*, and *dhaB3* identified as SEQ ID NOS:1, 2 and 3, respectively.

- 5 The *dhaB1*, *dhaB2*, and *dhaB3* genes code for the  $\alpha$ ,  $\beta$ , and  $\gamma$  subunits of the glycerol dehydratase enzyme, respectively.

The terms "oxidoreductase" or "1,3-propanediol oxidoreductase" refer to the polypeptide(s) responsible for an enzyme activity that is capable of catalyzing the reduction of 3-hydroxypropionaldehyde to 1,3-propanediol. 1,3-Propanediol oxidoreductase includes, for example, the polypeptide encoded by the *dhaT* gene (GenBank U09771, U30903) and is identified as SEQ ID NO:4.

The terms "glycerol-3-phosphate dehydrogenase" or "G3PDH" refer to the polypeptide(s) responsible for an enzyme activity capable of catalyzing the conversion of dihydroxyacetone phosphate (DHAP) to glycerol-3-phosphate (G3P). *In vivo* G3PDH may be NADH-, NADPH-, or FAD-dependent. Examples of this enzyme activity include the following: NADH-dependent enzymes (EC 1.1.1.8) are encoded by several genes including GPD1 (GenBank Z74071x2) or GPD2 (GenBank Z35169x1) or GPD3 (GenBank G984182) or DAR1 (GenBank Z74071x2); a NADPH-dependent enzyme (EC 1.1.1.94) is encoded by *gpsA* (GenBank U32164, G466746 (cds 197911-196892), and L45246); and FAD-dependent enzymes (EC 1.1.99.5) are encoded by GUT2 (GenBank Z47047x23) or glpD (GenBank G147838) or glpABC (GenBank M20938).

The terms "glycerol-3-phosphatase" or "sn-glycerol-3-phosphatase" or "d,l-glycerol phosphatase" or "G3P phosphatase" refer to the polypeptide(s) responsible for an enzyme activity that is capable of catalyzing the conversion of glycerol-3-phosphate to glycerol. G3P phosphatase includes, for example, the polypeptides encoded by GPP1 (GenBank Z47047x125) or GPP2 (GenBank U18813x11).

The term "glycerol kinase" refers to the polypeptide(s) responsible for an enzyme activity capable of catalyzing the conversion of glycerol to glycerol-3-phosphate or glycerol-3-phosphate to glycerol, depending on reaction conditions. Glycerol kinase includes, for example, the polypeptide encoded by GUT1 (GenBank U11583x19).

The terms "GPD1", "DAR1", "OSG1", "D2830", and "YDL022W" will be used interchangeably and refer to a gene that encodes a cytosolic glycerol-3-phosphate dehydrogenase and characterized by the base sequence given as SEQ ID NO:5.

The term "GPD2" refers to a gene that encodes a cytosolic glycerol-3-phosphate dehydrogenase and characterized by the base sequence given as SEQ ID NO:6.

5 The terms "GUT2" and "YIL155C" are used interchangeably and refer to a gene that encodes a mitochondrial glycerol-3-phosphate dehydrogenase and characterized by the base sequence given in SEQ ID NO:7.

The terms "GPP1", "RHR2" and "YIL053W" are used interchangeably and refer to a gene that encodes a cytosolic glycerol-3-phosphatase and characterized by the base sequence given as SEQ ID NO:8.

10 The terms "GPP2", "HOR2" and "YER062C" are used interchangeably and refer to a gene that encodes a cytosolic glycerol-3-phosphatase and characterized by the base sequence given as SEQ ID NO:9.

The term "GUT1" refers to a gene that encodes a cytosolic glycerol kinase and characterized by the base sequence given as SEQ ID NO:10.

15 The terms "function" or "enzyme function" refer to the catalytic activity of an enzyme in altering the energy required to perform a specific chemical reaction. It is understood that such an activity may apply to a reaction in equilibrium where the production of either product or substrate may be accomplished under suitable conditions.

20 The terms "polypeptide" and "protein" are used interchangeably.

The terms "carbon substrate" and "carbon source" refer to a carbon source capable of being metabolized by host organisms of the present invention and particularly carbon sources selected from the group consisting of monosaccharides, oligosaccharides, polysaccharides, and one-carbon substrates or  
25 mixtures thereof.

The terms "host cell" or "host organism" refer to a microorganism capable of receiving foreign or heterologous genes and of expressing those genes to produce an active gene product.

30 The terms "foreign gene", "foreign DNA", "heterologous gene" and "heterologous DNA" refer to genetic material native to one organism that has been placed within a host organism by various means.

The terms "recombinant organism" and "transformed host" refer to any organism having been transformed with heterologous or foreign genes. The recombinant organisms of the present invention express foreign genes encoding  
35 glycerol-3-phosphate dehydrogenase (G3PDH) and glycerol-3-phosphatase (G3P phosphatase), glycerol dehydratase (*dhaB*), and 1,3-propanediol oxidoreductase (*dhaT*) for the production of 1,3-propanediol from suitable carbon substrates.

"Gene" refers to a nucleic acid fragment that expresses a specific protein, including regulatory sequences preceding (5' non-coding) and following (3' non-coding) the coding region. The terms "native" and "wild-type" refer to a gene as found in nature with its own regulatory sequences.

5       The terms "encoding" and "coding" refer to the process by which a gene, through the mechanisms of transcription and translation, produces an amino acid sequence. It is understood that the process of encoding a specific amino acid sequence includes DNA sequences that may involve base changes that do not cause a change in the encoded amino acid, or which involve base changes which  
10       may alter one or more amino acids, but do not affect the functional properties of the protein encoded by the DNA sequence. It is therefore understood that the invention encompasses more than the specific exemplary sequences. Modifications to the sequence, such as deletions, insertions, or substitutions in the sequence which produce silent changes that do not substantially affect the  
15       functional properties of the resulting protein molecule are also contemplated. For example, alteration in the gene sequence which reflect the degeneracy of the genetic code, or which result in the production of a chemically equivalent amino acid at a given site, are contemplated. Thus, a codon for the amino acid alanine, a hydrophobic amino acid, may be substituted by a codon encoding another less  
20       hydrophobic residue, such as glycine, or a more hydrophobic residue, such as valine, leucine, or isoleucine. Similarly, changes which result in substitution of one negatively charged residue for another, such as aspartic acid for glutamic acid, or one positively charged residue for another, such as lysine for arginine, can also be expected to produce a biologically equivalent product. Nucleotide changes  
25       which result in alteration of the N-terminal and C-terminal portions of the protein molecule would also not be expected to alter the activity of the protein. In some cases, it may in fact be desirable to make mutants of the sequence in order to study the effect of alteration on the biological activity of the protein. Each of the proposed modifications is well within the routine skill in the art, as is  
30       determination of retention of biological activity in the encoded products. Moreover, the skilled artisan recognizes that sequences encompassed by this invention are also defined by their ability to hybridize, under stringent conditions (0.1X SSC, 0.1% SDS, 65 °C), with the sequences exemplified herein.

35       The term "expression" refers to the transcription and translation to gene product from a gene coding for the sequence of the gene product.

      The terms "plasmid", "vector", and "cassette" refer to an extra chromosomal element often carrying genes which are not part of the central metabolism of the cell, and usually in the form of circular double-stranded DNA

molecules. Such elements may be autonomously replicating sequences, genome integrating sequences, phage or nucleotide sequences, linear or circular, of a single- or double-stranded DNA or RNA, derived from any source, in which a number of nucleotide sequences have been joined or recombined into a unique construction which is capable of introducing a promoter fragment and DNA sequence for a selected gene product along with appropriate 3' untranslated sequence into a cell. "Transformation cassette" refers to a specific vector containing a foreign gene and having elements in addition to the foreign gene that facilitate transformation of a particular host cell. "Expression cassette" refers to a specific vector containing a foreign gene and having elements in addition to the foreign gene that allow for enhanced expression of that gene in a foreign host.

The terms "transformation" and "transfection" refer to the acquisition of new genes in a cell after the incorporation of nucleic acid. The acquired genes may be integrated into chromosomal DNA or introduced as extrachromosomal replicating sequences. The term "transformant" refers to the product of a transformation.

The term "genetically altered" refers to the process of changing hereditary material by transformation or mutation.

#### CONSTRUCTION OF RECOMBINANT ORGANISMS:

Recombinant organisms containing the necessary genes that will encode the enzymatic pathway for the conversion of a carbon substrate to 1,3-propanediol may be constructed using techniques well known in the art. In the present invention genes encoding glycerol-3-phosphate dehydrogenase (G3PDH), glycerol-3-phosphatase (G3P phosphatase), glycerol dehydratase (*dhaB*), and 1,3-propanediol oxidoreductase (*dhaT*) were isolated from a native host such as *Klebsiella* or *Saccharomyces* and used to transform host strains such as *E. coli* DH5 $\alpha$ , ECL707, AA200, or W1485; the *Saccharomycetes cerevisiae* strain YPH500; or the *Klebsiella pneumoniae* strains ATCC 25955 or ECL 2106.

#### Isolation of Genes

Methods of obtaining desired genes from a bacterial genome are common and well known in the art of molecular biology. For example, if the sequence of the gene is known, suitable genomic libraries may be created by restriction endonuclease digestion and may be screened with probes complementary to the desired gene sequence. Once the sequence is isolated, the DNA may be amplified using standard primer directed amplification methods such as polymerase chain reaction (PCR) (U.S. 4,683,202) to obtain amounts of DNA suitable for transformation using appropriate vectors.

Alternatively, cosmid libraries may be created where large segments of genomic DNA (35-45kb) may be packaged into vectors and used to transform appropriate hosts. Cosmid vectors are unique in being able to accommodate large quantities of DNA. Generally, cosmid vectors have at least one copy of the *cos* DNA sequence which is needed for packaging and subsequent circularization of the foreign DNA. In addition to the *cos* sequence these vectors will also contain an origin of replication such as ColE1 and drug resistance markers such as a gene resistant to ampicillin or neomycin. Methods of using cosmid vectors for the transformation of suitable bacterial hosts are well described in Sambrook et al., Molecular Cloning: A Laboratory Manual, Second Edition (1989) Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY (1989).

Typically to clone cosmids, foreign DNA is isolated and ligated, using the appropriate restriction endonucleases, adjacent to the *cos* region of the cosmid vector. Cosmid vectors containing the linearized foreign DNA is then reacted with a DNA packaging vehicle such as bacteriophage  $\lambda$ . During the packaging process the *cos* sites are cleaved and the foreign DNA is packaged into the head portion of the bacterial viral particle. These particles are then used to transfect suitable host cells such as *E. coli*. Once injected into the cell, the foreign DNA circularizes under the influence of the *cos* sticky ends. In this manner large segments of foreign DNA can be introduced and expressed in recombinant host cells.

Isolation and cloning of genes encoding glycerol dehydratase (*dhaB*) and 1,3-propanediol oxidoreductase (*dhaT*)

Cosmid vectors and cosmid transformation methods were used within the context of the present invention to clone large segments of genomic DNA from bacterial genera known to possess genes capable of processing glycerol to 1,3-propanediol. Specifically, genomic DNA from *K. pneumoniae* ATCC 25955 was isolated by methods well known in the art and digested with the restriction enzyme Sau3A for insertion into a cosmid vector Supercos 1 and packaged using GigapackII packaging extracts. Following construction of the vector *E. coli* XL1-Blue MR cells were transformed with the cosmid DNA. Transformants were screened for the ability to convert glycerol to 1,3-propanediol by growing the cells in the presence of glycerol and analyzing the media for 1,3-propanediol formation.

Two of the 1,3-propanediol positive transformants were analyzed and the cosmids were named pKP1 and pKP2. DNA sequencing revealed extensive homology to the glycerol dehydratase gene (*dhaB*) from *C. freundii*, demonstrating that these transformants contained DNA encoding the glycerol dehydratase gene. Other 1,3-propanediol positive transformants were analyzed

and the cosmids were named pKP4 and pKP5. DNA sequencing revealed that these cosmids carried DNA encoding a diol dehydratase gene.

Although the instant invention utilizes the isolated genes from within a *Klebsiella* cosmid, alternate sources of dehydratase genes include, but are not limited to, *Citrobacter*, *Clostridia*, and *Salmonella*.

Genes encoding G3PDH and G3P phosphatase

The present invention provides genes suitable for the expression of G3PDH and G3P phosphatase activities in a host cell.

Genes encoding G3PDH are known. For example, GPD1 has been isolated from *Saccharomyces* and has the base sequence given by SEQ ID NO:5, encoding the amino acid sequence given in SEQ ID NO:11 (Wang et al., *supra*). Similarly, G3PDH activity has also been isolated from *Saccharomyces* encoded by GPD2 having the base sequence given in SEQ ID NO:6, encoding the amino acid sequence given in SEQ ID NO:12 (Eriksson et al., *Mol. Microbiol.* 17, 95, (1995).

It is contemplated that any gene encoding a polypeptide responsible for G3PDH activity is suitable for the purposes of the present invention wherein that activity is capable of catalyzing the conversion of dihydroxyacetone phosphate (DHAP) to glycerol-3-phosphate (G3P). Further, it is contemplated that any gene encoding the amino acid sequence of G3PDH as given by any one of SEQ ID NOS:11, 12, 13, 14, 15 and 16 corresponding to the genes GPD1, GPD2, GUT2, gpsA, glpD, and the  $\alpha$  subunit of glpABC, respectively, will be functional in the present invention wherein that amino acid sequence encompasses amino acid substitutions, deletions or additions that do not alter the function of the enzyme. It will be appreciated by the skilled person that genes encoding G3PDH isolated from other sources are also be suitable for use in the present invention. For example, genes isolated from prokaryotes include GenBank accessions M34393, M20938, L06231, U12567, L45246, L45323, L45324, L45325, U32164, and U39682; genes isolated from fungi include GenBank accessions U30625, U30876 and X56162; genes isolated from insects include GenBank accessions X61223 and X14179; and genes isolated from mammalian sources include GenBank accessions U12424, M25558 and X78593.

Genes encoding G3P phosphatase are known. For example, GPP2 has been isolated from *Saccharomyces cerevisiae* and has the base sequence given by SEQ ID NO:9 which encodes the amino acid sequence given in SEQ ID NO:17 (Norbeck et al., *J. Biol. Chem.* 271, p. 13875, 1996).

It is contemplated that any gene encoding a G3P phosphatase activity is suitable for the purposes of the present invention wherein that activity is capable



of catalyzing the conversion of glycerol-3-phosphate to glycerol. Further, it is contemplated that any gene encoding the amino acid sequence of G3P phosphatase as given by SEQ ID NOS:33 and 17 will be functional in the present invention wherein that amino acid sequence encompasses amino acid

- 5 substitutions, deletions or additions that do not alter the function of the enzyme. It will be appreciated by the skilled person that genes encoding G3P phosphatase isolated from other sources are also suitable for use in the present invention. For example, the dephosphorylation of glycerol-3-phosphate to yield glycerol may be achieved with one or more of the following general or specific phosphatases:
- 10 alkaline phosphatase (EC 3.1.3.1) [GenBank M19159, M29663, U02550 or M33965]; acid phosphatase (EC 3.1.3.2) [GenBank U51210, U19789, U28658 or L20566]; glycerol-3-phosphatase (EC 3.1.3.-) [GenBank Z38060 or U18813x11]; glucose-1-phosphatase (EC 3.1.3.10) [GenBank M33807]; glucose-6-phosphatase (EC 3.1.3.9) [GenBank U00445]; fructose-1,6-bisphosphatase (EC 3.1.3.11)
- 15 [GenBank X12545 or J03207] or phosphatidyl glycerol phosphate phosphatase (EC 3.1.3.27) [GenBank M23546 and M23628].

- Genes encoding glycerol kinase are known. For example, GUT1 encoding the glycerol kinase from *Saccharaomyces* has been isolated and sequenced (Pavlik et al., *Curr. Genet.* 24, 21, (1993)) and the base sequence is given by SEQ ID
- 20 NO:10 which encodes the amino acid sequence given in SEQ ID NO:18. It will be appreciated by the skilled artisan that although glycerol kinase catalyzes the degradation of glycerol in nature the same enzyme will be able to function in the synthesis of glycerol to convert glycerol-3-phosphate to glycerol under the appropriate reaction energy conditions. Evidence exists for glycerol production
- 25 through a glycerol kinase. Under anaerobic or respiration-inhibited conditions, *Trypanosoma brucei* gives rise to glycerol in the presence of Glycerol-3-P and ADP. The reaction occurs in the glycosome compartment (D. Hammond, *J. Biol. Chem.* 260, 15646-15654, (1985)).

#### Host cells

- 30 Suitable host cells for the recombinant production of glycerol by the expression of G3PDH and G3P phosphatase may be either prokaryotic or eukaryotic and will be limited only by their ability to express active enzymes. Preferred hosts will be those typically useful for production of glycerol or 1,3-propanediol such as *Citrobacter*, *Enterobacter*, *Clostridium*, *Klebsiella*,
- 35 *Aerobacter*, *Lactobacillus*, *Aspergillus*, *Saccharomyces*, *Schizosaccharomyces*, *Zygosaccharomyces*, *Pichia*, *Kluyveromyces*, *Candida*, *Hansenula*, *Debaryomyces*, *Mucor*, *Torulopsis*, *Methylobacter*, *Escherichia*, *Salmonella*,

*Bacillus*, *Streptomyces* and *Pseudomonas*. Most preferred in the present invention are *E. coli*, *Klebsiella* species and *Saccharomyces* species.

Adenosyl-cobalamin (coenzyme B<sub>12</sub>) is an essential cofactor for glycerol dehydratase activity. The coenzyme is the most complex non-polymeric natural product known, and its synthesis *in vivo* is directed using the products of about 30 genes. Synthesis of coenzyme B<sub>12</sub> is found in prokaryotes, some of which are able to synthesize the compound *de novo*, while others can perform partial reactions. *E. coli*, for example, cannot fabricate the corrin ring structure, but is able to catalyse the conversion of cobinamide to corrinoid and can introduce the 5'-deoxyadenosyl group.

Eukaryotes are unable to synthesize coenzyme B<sub>12</sub> *de novo* and instead transport vitamin B<sub>12</sub> from the extracellular milieu with subsequent conversion of the compound to its functional form of the compound by cellular enzymes. Three enzyme activities have been described for this series of reactions.

1) aquacobalamin reductase (EC 1.6.99.8) reduces Co(III) to Co(II);  
2) cob(II)alamin reductase (EC 1.6.99.9) reduces Co(II) to Co(I); and  
3) cob(I)alamin adenosyltransferase (EC 2.5.1.17) transfers a 5'-deoxyadenosine moiety from ATP to the reduced corrinoid. This last enzyme activity is the best characterized of the three, and is encoded by *cobA* in *S. typhimurium*, *btuR* in *E. coli* and *cobO* in *P. denitrificans*. These three cob(I)alamin adenosyltransferase genes have been cloned and sequenced. Cob(I)alamin adenosyltransferase activity has been detected in human fibroblasts and in isolated rat mitochondria (Fenton et al., *Biochem. Biophys. Res. Commun.* 98, 283-9, (1981)). The two enzymes involved in cobalt reduction are poorly characterized and gene sequences are not available. There are reports of an aquacobalamin reductase from *Euglena gracilis* (Watanabe et al., *Arch. Biochem. Biophys.* 305, 421-7, (1993)) and a microsomal cob(III)alamin reductase is present in the microsomal and mitochondrial inner membrane fractions from rat fibroblasts (Pezacka, *Biochim. Biophys. Acta*, 1157, 167-77, (1993)).

Supplementing culture media with vitamin B<sub>12</sub> may satisfy the need to produce coenzyme B<sub>12</sub> for glycerol dehydratase activity in many microorganisms, but in some cases additional catalytic activities may have to be added or increased *in vivo*. Enhanced synthesis of coenzyme B<sub>12</sub> in eukaryotes may be particularly desirable. Given the published sequences for genes encoding cob(I)alamin adenosyltransferase, the cloning and expression of this gene could be accomplished by one skilled in the art. For example, it is contemplated that yeast, such as *Saccharomyces*, could be constructed so as to contain genes encoding cob(I)alamin adenosyltransferase in addition to the genes necessary to effect

conversion of a carbon substrate such as glucose to 1,3-propanediol. Cloning and expression of the genes for cobalt reduction requires a different approach. This could be based on a selection in *E. coli* for growth on ethanolamine as sole N<sub>2</sub> source. In the presence of coenzyme B<sub>12</sub> ethanolamine ammonia-lyase enables growth of cells in the absence of other N<sub>2</sub> sources. If *E. coli* cells contain a cloned gene for cob(I)alamin adenosyltransferase and random cloned DNA from another organism, growth on ethanolamine in the presence of aquacobalamin should be enhanced and selected for if the random cloned DNA encodes cobalt reduction properties to facilitate adenosylation of aquacobalamin.

In addition to *E. coli* and *Saccharomyces*, *Klebsiella* is a particularly preferred host. Strains of *Klebsiella pneumoniae* are known to produce 1,3-propanediol when grown on glycerol as the sole carbon. It is contemplated that *Klebsiella* can be genetically altered to produce 1,3-propanediol from monosaccharides, oligosaccharides, polysaccharides, or one-carbon substrates.

In order to engineer such strains, it will be advantageous to provide the *Klebsiella* host with the genes facilitating conversion of dihydroxyacetone phosphate to glycerol and conversion of glycerol to 1,3-propanediol either separately or together, under the transcriptional control of one or more constitutive or inducible promoters. The introduction of the DAR1 and GPP2 genes encoding glycerol-3-phosphate dehydrogenase and glycerol-3-phosphatase, respectively, will provide *Klebsiella* with genetic machinery to produce 1,3-propanediol from an appropriate carbon substrate.

The genes (e.g., G3PDH, G3P phosphatase, *dhaB* and/or *dhaT*) may be introduced on any plasmid vector capable of replication in *K. pneumoniae* or they may be integrated into the *K. pneumoniae* genome. For example, *K. pneumoniae* ATCC 25955 and *K. pneumoniae* ECL 2106 are known to be sensitive to tetracycline or chloramphenicol; thus plasmid vectors which are both capable of replicating in *K. pneumoniae* and encoding resistance to either or both of these antibiotics may be used to introduce these genes into *K. pneumoniae*. Methods of transforming *Klebsiella* with genes of interest are common and well known in the art and suitable protocols, including appropriate vectors and expression techniques may be found in Sambrook, *supra*.

#### Vectors and expression cassettes

The present invention provides a variety of vectors and transformation and expression cassettes suitable for the cloning, transformation and expression of G3PDH and G3P phosphatase into a suitable host cell. Suitable vectors will be those which are compatible with the bacterium employed. Suitable vectors can be derived, for example, from a bacteria, a virus (such as bacteriophage T7 or a M-13

derived phage), a cosmid, a yeast or a plant. Protocols for obtaining and using such vectors are known to those in the art. (Sambrook et al., Molecular Cloning: A Laboratory Manual - volumes 1,2,3 (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, (1989)).

- 5 Typically, the vector or cassette contains sequences directing transcription and translation of the relevant gene, a selectable marker, and sequences allowing autonomous replication or chromosomal integration. Suitable vectors comprise a region 5' of the gene which harbors transcriptional initiation controls and a region 3' of the DNA fragment which controls transcriptional termination. It is most  
10 preferred when both control regions are derived from genes homologous to the transformed host cell although it is to be understood that such control regions need not be derived from the genes native to the specific species chosen as a production host.

- Initiation control regions or promoters, which are useful to drive  
15 expression of the G3PDH and G3P phosphatase genes in the desired host cell, are numerous and familiar to those skilled in the art. Virtually any promoter capable of driving these genes is suitable for the present invention including but not limited to CYC1, HIS3, GAL1, GAL10, ADH1, PGK, PHO5, GAPDH, ADC1, TRP1, URA3, LEU2, ENO, TPI (useful for expression in *Saccharomyces*); AOX1  
20 (useful for expression in *Pichia*); and lac, trp,  $\lambda P_L$ ,  $\lambda P_R$ , T7, tac, and trc (useful for expression in *E. coli*).

- Termination control regions may also be derived from various genes native to the preferred hosts. Optionally, a termination site may be unnecessary, however, it is most preferred if included.

- 25 For effective expression of the instant enzymes, DNA encoding the enzymes are linked operably through initiation codons to selected expression control regions such that expression results in the formation of the appropriate messenger RNA.

- Transformation of suitable hosts and expression of genes for the  
30 production of 1,3-propanediol

- Once suitable cassettes are constructed they are used to transform appropriate host cells. Introduction of the cassette containing the genes encoding glycerol-3-phosphate dehydrogenase (G3PDH) and glycerol-3-phosphatase (G3P phosphatase), glycerol dehydratase (*dhaB*), and 1,3-propanediol oxidoreductase  
35 (*dhaT*), either separately or together into the host cell may be accomplished by known procedures such as by transformation (e.g., using calcium-permeabilized cells, electroporation) or by transfection using a recombinant phage virus. (Sambrook et al., *supra*.)

In the present invention, *E. coli* W2042 (ATCC 98188) containing the genes encoding glycerol-3-phosphate dehydrogenase (G3PDH) and glycerol-3-phosphatase (G3P phosphatase), glycerol dehydratase (*dhaB*), and 1,3-propanediol oxidoreductase (*dhaT*) was created. Additionally, *S. cerevisiae* YPH500  
5 (ATCC 74392) harboring plasmids pMCK10, pMCK17, pMCK30 and pMCK35 containing genes encoding glycerol-3-phosphate dehydrogenase (G3PDH) and glycerol-3-phosphatase (G3P phosphatase), glycerol dehydratase (*dhaB*), and 1,3-propanediol oxidoreductase (*dhaT*) was constructed. Both the above-mentioned transformed *E. coli* and *Saccharomyces* represent preferred  
10 embodiments of the invention.

Media and Carbon Substrates:

Fermentation media in the present invention must contain suitable carbon substrates. Suitable substrates may include but are not limited to monosaccharides such as glucose and fructose, oligosaccharides such as lactose or sucrose,  
15 polysaccharides such as starch or cellulose, or mixtures thereof, and unpurified mixtures from renewable feedstocks such as cheese whey permeate, cornsteep liquor, sugar beet molasses, and barley malt. Additionally, the carbon substrate may also be one-carbon substrates such as carbon dioxide, or methanol for which metabolic conversion into key biochemical intermediates has been demonstrated.

20 Glycerol production from single carbon sources (e.g., methanol, formaldehyde, or formate) has been reported in methylotrophic yeasts (Yamada et al., *Agric. Biol. Chem.*, 53(2) 541-543, (1989)) and in bacteria (Hunter et.al., *Biochemistry*, 24, 4148-4155, (1985)). These organisms can assimilate single carbon compounds, ranging in oxidation state from methane to formate, and  
25 produce glycerol. The pathway of carbon assimilation can be through ribulose monophosphate, through serine, or through xylulose-momophosphate (Gottschalk, Bacterial Metabolism, Second Edition, Springer-Verlag: New York (1986)). The ribulose monophosphate pathway involves the condensation of formate with ribulose-5-phosphate to form a 6 carbon sugar that becomes fructose and  
30 eventually the three carbon product glyceraldehyde-3-phosphate. Likewise, the serine pathway assimilates the one-carbon compound into the glycolytic pathway via methylenetetrahydrofolate.

In addition to utilization of one and two carbon substrates, methylotrophic organisms are also known to utilize a number of other carbon-containing  
35 compounds such as methylamine, glucosamine and a variety of amino acids for metabolic activity. For example, methylotrophic yeast are known to utilize the carbon from methylamine to form trehalose or glycerol (Bellion et al., *Microb. Growth C1 Compd.*, [Int. Symp.], 7th (1993), 415-32. Editor(s): Murrell, J.

Collin; Kelly, Don P. Publisher: Intercept, Andover, UK). Similarly, various species of *Candida* will metabolize alanine or oleic acid (Sulter et al., *Arch. Microbiol.*, 153(5), 485-9 (1990)). Hence, the source of carbon utilized in the present invention may encompass a wide variety of carbon-containing substrates and will only be limited by the requirements of the host organism.

Although it is contemplated that all of the above mentioned carbon substrates and mixtures thereof are suitable in the present invention, preferred carbon substrates are monosaccharides, oligosaccharides, polysaccharides, and one-carbon substrates. More preferred are sugars such as glucose, fructose, sucrose and single carbon substrates such as methanol and carbon dioxide. Most preferred is glucose.

In addition to an appropriate carbon source, fermentation media must contain suitable minerals, salts, cofactors, buffers and other components, known to those skilled in the art, suitable for the growth of the cultures and promotion of the enzymatic pathway necessary for glycerol production. Particular attention is given to Co(II) salts and/or vitamin B<sub>12</sub> or precursors thereof.

Culture Conditions:

Typically, cells are grown at 30 °C in appropriate media. Preferred growth media in the present invention are common commercially prepared media such as Luria Bertani (LB) broth, Sabouraud Dextrose (SD) broth or Yeast Malt Extract (YM) broth. Other defined or synthetic growth media may also be used and the appropriate medium for growth of the particular microorganism will be known by someone skilled in the art of microbiology or fermentation science. The use of agents known to modulate catabolite repression directly or indirectly, e.g., cyclic adenosine 2':3'-monophosphate or cyclic adenosine 2':5'-monophosphate, may also be incorporated into the reaction media. Similarly, the use of agents known to modulate enzymatic activities (e.g., sulphites, bisulphites and alkalis) that lead to enhancement of glycerol production may be used in conjunction with or as an alternative to genetic manipulations.

Suitable pH ranges for the fermentation are between pH 5.0 to pH 9.0, where pH 6.0 to pH 8.0 is preferred as range for the the initial condition.

Reactions may be performed under aerobic or anaerobic conditions where anaerobic or microaerobic conditions are preferred.

Batch and Continuous Fermentations:

The present process uses a batch method of fermentation. A classical batch fermentation is a closed system where the composition of the media is set at the beginning of the fermentation and not subject to artificial alterations during the fermentation. Thus, at the beginning of the fermentation the media is inoculated

with the desired organism or organisms and fermentation is permitted to occur adding nothing to the system. Typically, however, a batch fermentation is "batch" with respect to the addition of the carbon source and attempts are often made at controlling factors such as pH and oxygen concentration. The metabolite and biomass compositions of the batch system change constantly up to the time the fermentation is stopped. Within batch cultures cells moderate through a static lag phase to a high growth log phase and finally to a stationary phase where growth rate is diminished or halted. If untreated, cells in the stationary phase will eventually die. Cells in log phase generally are responsible for the bulk of production of end product or intermediate.

A variation on the standard batch system is the Fed-Batch fermentation system which is also suitable in the present invention. In this variation of a typical batch system, the substrate is added in increments as the fermentation progresses. Fed-Batch systems are useful when catabolite repression is apt to inhibit the metabolism of the cells and where it is desirable to have limited amounts of substrate in the media. Measurement of the actual substrate concentration in Fed-Batch systems is difficult and is therefore estimated on the basis of the changes of measurable factors such as pH, dissolved oxygen and the partial pressure of waste gases such as CO<sub>2</sub>. Batch and Fed-Batch fermentations are common and well known in the art and examples may be found in Brock, *supra*.

It is also contemplated that the method would be adaptable to continuous fermentation methods. Continuous fermentation is an open system where a defined fermentation media is added continuously to a bioreactor and an equal amount of conditioned media is removed simultaneously for processing. Continuous fermentation generally maintains the cultures at a constant high density where cells are primarily in log phase growth.

Continuous fermentation allows for the modulation of one factor or any number of factors that affect cell growth or end product concentration. For example, one method will maintain a limiting nutrient such as the carbon source or nitrogen level at a fixed rate and allow all other parameters to moderate. In other systems a number of factors affecting growth can be altered continuously while the cell concentration, measured by media turbidity, is kept constant. Continuous systems strive to maintain steady state growth conditions and thus the cell loss due to media being drawn off must be balanced against the cell growth rate in the fermentation. Methods of modulating nutrients and growth factors for continuous fermentation processes as well as techniques for maximizing the rate

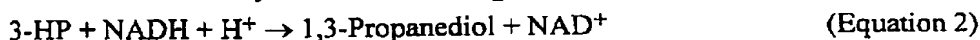
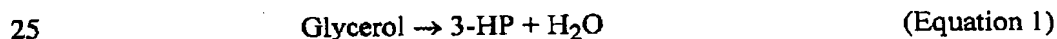
of product formation are well known in the art of industrial microbiology and a variety of methods are detailed by Brock, *supra*.

The present invention may be practiced using either batch, fed-batch or continuous processes and that any known mode of fermentation would be suitable.

- 5 Additionally, it is contemplated that cells may be immobilized on a substrate as whole cell catalysts and subjected to fermentation conditions for 1,3-propanediol production.

Alterations in the 1,3-propanediol production pathway:

- 10 Representative enzyme pathway. The production of 1,3-propanediol from glucose can be accomplished by the following series of steps. This series is representative of a number of pathways known to those skilled in the art. Glucose is converted in a series of steps by enzymes of the glycolytic pathway to dihydroxyacetone phosphate (DHAP) and 3-phosphoglyceraldehyde (3-PG). Glycerol is then formed by either hydrolysis of DHAP to dihydroxyacetone
- 15 (DHA) followed by reduction, or reduction of DHAP to glycerol 3-phosphate (G3P) followed by hydrolysis. The hydrolysis step can be catalyzed by any number of cellular phosphatases which are known to be specific or non-specific with respect to their substrates or the activity can be introduced into the host by recombination. The reduction step can be catalyzed by a NAD<sup>+</sup> (or NADP<sup>+</sup>)
- 20 linked host enzyme or the activity can be introduced into the host by recombination. It is notable that the *dha* regulon contains a glycerol dehydrogenase (E.C. 1.1.1.6) which catalyzes the reversible reaction of Equation 3.



- 30 Glycerol is converted to 1,3-propanediol via the intermediate 3-hydroxy-propionaldehyde (3-HP) as has been described in detail above. The intermediate 3-HP is produced from glycerol (Equation 1) by a dehydratase enzyme which can be encoded by the host or can introduced into the host by recombination. This dehydratase can be glycerol dehydratase (E.C. 4.2.1.30), diol dehydratase (E.C. 4.2.1.28), or any other enzyme able to catalyze this transformation.
- 35 Glycerol dehydratase, but not diol dehydratase, is encoded by the *dha* regulon. 1,3-Propanediol is produced from 3-HP (Equation 2) by a NAD<sup>+</sup>- (or NADP<sup>+</sup>) linked host enzyme or the activity can introduced into the host by recombination. This final reaction in the production of 1,3-propanediol can be catalyzed by 1,3-propanediol dehydrogenase (E.C. 1.1.1.202) or other alcohol dehydrogenases.



Mutations and transformations that affect carbon channeling. A variety of mutant organisms comprising variations in the 1,3-propanediol production pathway will be useful in the present invention. The introduction of a triosephosphate isomerase mutation (*tpi*-) into the microorganism is an example of the use of a mutation to improve the performance by carbon channeling. Alternatively, mutations which diminish the production of ethanol (*adh*) or lactate (*ldh*) will increase the availability of NADH for the production of 1,3-propanediol. Additional mutations in steps of glycolysis after glyceraldehyde-3-phosphate such as phosphoglycerate mutase (*pgm*) would be useful to increase the flow of carbon to the 1,3-propanediol production pathway. Mutations that effect glucose transport such as PTS which would prevent loss of PEP may also prove useful. Mutations which block alternate pathways for intermediates of the 1,3-propanediol production pathway such as the glycerol catabolic pathway (*glp*) would also be useful to the present invention. The mutation can be directed toward a structural gene so as to impair or improve the activity of an enzymatic activity or can be directed toward a regulatory gene so as to modulate the expression level of an enzymatic activity.

Alternatively, transformations and mutations can be combined so as to control particular enzyme activities for the enhancement of 1,3-propanediol production. Thus it is within the scope of the present invention to anticipate modifications of a whole cell catalyst which lead to an increased production of 1,3-propanediol.

Identification and purification of 1,3-propanediol:

Methods for the purification of 1,3-propanediol from fermentation media are known in the art. For example, propanediols can be obtained from cell media by subjecting the reaction mixture to extraction with an organic solvent, distillation and column chromatography (U.S. 5,356,812). A particularly good organic solvent for this process is cyclohexane (U.S. 5,008,473).

1,3-Propanediol may be identified directly by submitting the media to high pressure liquid chromatography (HPLC) analysis. Preferred in the present invention is a method where fermentation media is analyzed on an analytical ion exchange column using a mobile phase of 0.01 N sulfuric acid in an isocratic fashion.

Identification and purification of G3PDH and G3P phosphatase:

The levels of expression of the proteins G3PDH and G3P phosphatase are measured by enzyme assays, G3PDH activity assay relied on the spectral properties of the cosubstrate, NADH, in the DHAP conversion to G-3-P. NADH has intrinsic UV/vis absorption and its consumption can be monitored

spectrophotometrically at 340 nm. G3P phosphatase activity can be measured by any method of measuring the inorganic phosphate liberated in the reaction. The most commonly used detection method used the visible spectroscopic determination of a blue-colored phosphomolybdate ammonium complex.

5

## EXAMPLES

### GENERAL METHODS

Procedures for phosphorylations, ligations and transformations are well known in the art. Techniques suitable for use in the following examples may be found in Sambrook, J. et al., Molecular Cloning: A Laboratory Manual, Second Edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY (1989).

Materials and methods suitable for the maintenance and growth of bacterial cultures are well known in the art. Techniques suitable for use in the following examples may be found as set out in Manual of Methods for General Bacteriology (Phillipp Gerhardt, R. G. E. Murray, Ralph N. Costilow, Eugene W. Nester, Willis A. Wood, Noel R. Krieg and G. Briggs Phillips, eds), American Society for Microbiology, Washington, DC. (1994)) or by Thomas D. Brock in Biotechnology: A Textbook of Industrial Microbiology, Second Edition, Sinauer Associates, Inc., Sunderland, MA (1989). All reagents and materials used for the growth and maintenance of bacterial cells were obtained from Aldrich Chemicals (Milwaukee, WI), DIFCO Laboratories (Detroit, MI), GIBCO/BRL (Gaithersburg, MD), or Sigma Chemical Company (St. Louis, MO) unless otherwise specified.

The meaning of abbreviations is as follows: "h" means hour(s), "min" means minute(s), "sec" means second(s), "d" means day(s), "mL" means milliliters, "L" means liters.

### 25 ENZYME ASSAYS

Glycerol dehydratase activity in cell-free extracts was determined using 1,2-propanediol as substrate. The assay, based on the reaction of aldehydes with methylbenzo-2-thiazolone hydrazone, has been described by Forage and Foster (*Biochim. Biophys. Acta*, 569, 249 (1979)). The activity of 1,3-propanediol oxidoreductase, sometimes referred to as 1,3-propanediol dehydrogenase, was determined in solution or in slab gels using 1,3-propanediol and NAD<sup>+</sup> as substrates as has also been described. Johnson and Lin, *J. Bacteriol.*, 169, 2050 (1987). NADH or NADPH dependent glycerol 3-phosphate dehydrogenase (G3PDH) activity was determined spectrophotometrically, following the disappearance of NADH or NADPH as has been described. (R. M. Bell and J. E. Cronan, Jr., *J. Biol. Chem.* 250:7153-8 (1975)).

#### Assay for glycerol-3-phosphatase, GPP

The assay for enzyme activity was performed by incubating the extract with an organic phosphate substrate in a bis-Tris or MES and magnesium buffer, pH 6.5. The substrate used was l- $\alpha$ -glycerol phosphate; d,l- $\alpha$ -glycerol phosphate.

5 The final concentrations of the reagents in the assay are: buffer (20 mM, bis-Tris or 50 mM MES);  $\text{MgCl}_2$  (10 mM); and substrate (20 mM). If the total protein in the sample was low and no visible precipitation occurs with an acid quench, the sample was conveniently assayed in the cuvette. This method involved incubating an enzyme sample in a cuvette that contained 20 mM substrate (50  $\mu\text{L}$ , 200 mM),

10 50 mM MES, 10 mM  $\text{MgCl}_2$ , pH 6.5 buffer. The final phosphatase assay volume was 0.5 mL. The enzyme-containing sample was added to the reaction mixture; the contents of the cuvette were mixed and then the cuvette was placed in a circulating water bath at  $T = 37^\circ\text{C}$  for 5 to 120 min – depending on whether the phosphatase activity in the enzyme sample ranged from 2 to 0.02 U/mL. The

15 enzymatic reaction was quenched by the addition of the acid molybdate reagent (0.4 mL). After the Fiske SubbaRow reagent (0.1 mL) and distilled water (1.5 mL) were added, the solution was mixed and allowed to develop. After 10 min, the absorbance of the samples was read at 660 nm using a Cary 219 UV/Vis spectrophotometer. The amount of inorganic phosphate released was

20 compared to a standard curve that was prepared by using a stock inorganic phosphate solution (0.65 mM) and preparing 6 standards with final inorganic phosphate concentrations ranging from 0.026 to 0.130  $\mu\text{mol/mL}$ .

#### Isolation and Identification 1,3-propanediol

The conversion of glycerol to 1,3-propanediol was monitored by HPLC.

25 Analyses were performed using standard techniques and materials available to one skilled in the art of chromatography. One suitable method utilized a Waters Maxima 820 HPLC system using UV (210 nm) and RI detection. Samples were injected onto a Shodex SH-1011 column (8 mm x 300 mm, purchased from Waters, Milford, MA) equipped with a Shodex SH-1011P precolumn (6 mm x

30 50 mm), temperature controlled at  $50^\circ\text{C}$ , using 0.01 N  $\text{H}_2\text{SO}_4$  as mobile phase at a flow rate of 0.5 mL/min. When quantitative analysis was desired, samples were prepared with a known amount of trimethylacetic acid as external standard. Typically, the retention times of glycerol (RI detection), 1,3-propanediol (RI detection), and trimethylacetic acid (UV and RI detection) were 20.67 min,

35 26.08 min, and 35.03 min, respectively.

Production of 1,3-propanediol was confirmed by GC/MS. Analyses were performed using standard techniques and materials available to one of skill in the art of GC/MS. One suitable method utilized a Hewlett Packard 5890 Series II gas

chromatograph coupled to a Hewlett Packard 5971 Series mass selective detector (EI) and a HP-INNOWax column (30 m length, 0.25 mm i.d., 0.25 micron film thickness). The retention time and mass spectrum of 1,3-propanediol generated were compared to that of authentic 1,3-propanediol (*m/e*: 57, 58).

- 5        An alternative method for GC/MS involved derivatization of the sample. To 1.0 mL of sample (e.g., culture supernatant) was added 30  $\mu$ L of concentrated (70% v/v) perchloric acid. After mixing, the sample was frozen and lyophilized. A 1:1 mixture of bis(trimethylsilyl)trifluoroacetamide:pyridine (300  $\mu$ L) was added to the lyophilized material, mixed vigorously and placed at 65 °C for one h.
- 10      The sample was clarified of insoluble material by centrifugation. The resulting liquid partitioned into two phases, the upper of which was used for analysis. The sample was chromatographed on a DB-5 column (48 m, 0.25 mm I.D., 0.25  $\mu$ m film thickness; from J&W Scientific) and the retention time and mass spectrum of the 1,3-propanediol derivative obtained from culture supernatants were compared
- 15      to that obtained from authentic standards. The mass spectrum of TMS-derivatized 1,3-propanediol contains the characteristic ions of 205, 177, 130 and 115 AMU.

#### EXAMPLE 1

#### CLONING AND TRANSFORMATION OF *E. COLI* HOST CELLS WITH COSMID DNA FOR THE EXPRESSION OF 1,3-PROPANEDIOL

##### 20      Media

- Synthetic S12 medium was used in the screening of bacterial transformants for the ability to make 1,3-propanediol. S12 medium contains: 10 mM ammonium sulfate, 50 mM potassium phosphate buffer, pH 7.0, 2 mM  $MgCl_2$ , 0.7 mM  $CaCl_2$ , 50  $\mu$ M  $MnCl_2$ , 1  $\mu$ M  $FeCl_3$ , 1  $\mu$ M  $ZnCl_2$ , 1.7  $\mu$ M  $CuSO_4$ , 2.5  $\mu$ M
- 25       $CoCl_2$ , 2.4  $\mu$ M  $Na_2MoO_4$ , and 2  $\mu$ M thiamine hydrochloride.

- Medium A used for growth and fermentation consisted of: 10 mM ammonium sulfate; 50 mM MOPS/KOH buffer, pH 7.5; 5 mM potassium phosphate buffer, pH 7.5; 2 mM  $MgCl_2$ ; 0.7 mM  $CaCl_2$ ; 50  $\mu$ M  $MnCl_2$ ; 1  $\mu$ M  $FeCl_3$ ; 1  $\mu$ M  $ZnCl_2$ ; 1.72  $\mu$ M  $CuSO_4$ ; 2.53  $\mu$ M  $CoCl_2$ ; 2.42  $\mu$ M  $Na_2MoO_4$ ; 2  $\mu$ M
- 30      thiamine hydrochloride; 0.01% yeast extract; 0.01% casamino acids; 0.8  $\mu$ g/mL vitamin B<sub>12</sub>; and 50  $\mu$ g/mL amp. Medium A was supplemented with either 0.2% glycerol or 0.2% glycerol plus 0.2% D-glucose as required.

##### Cells:

- Klebsiella pneumoniae* ECL2106 (Ruch et al., *J. Bacteriol.*, 124, 348
- 35      (1975)), also known in the literature as *K. aerogenes* or *Aerobacter aerogenes*, was obtained from E. C. C. Lin (Harvard Medical School, Cambridge, MA) and was maintained as a laboratory culture.

*Klebsiella pneumoniae* ATCC 25955 was purchased from American Type Culture Collection (Rockville, MD).

5 *E. coli* DH5 $\alpha$  was purchased from Gibco/BRL and was transformed with the cosmid DNA isolated from *Klebsiella pneumoniae* ATCC 25955 containing a gene coding for either a glycerol or diol dehydratase enzyme. Cosmids containing the glycerol dehydratase were identified as pKP1 and pKP2 and cosmid containing the diol dehydratase enzyme were identified as pKP4. Transformed DH5 $\alpha$  cells were identified as DH5 $\alpha$ -pKP1, DH5 $\alpha$ -pKP2, and DH5 $\alpha$ -pKP4.

10 *E. coli* ECL707 (Sprenger et al., *J. Gen. Microbiol.*, 135, 1255 (1989)) was obtained from E. C. C. Lin (Harvard Medical School, Cambridge, MA) and was similarly transformed with cosmid DNA from *Klebsiella pneumoniae*. These transformants were identified as ECL707-pKP1 and ECL707-pKP2, containing the glycerol dehydratase gene and ECL707-pKP4 containing the diol dehydratase gene.

15 *E. coli* AA200 containing a mutation in the *tpi* gene (Anderson et al., *J. Gen. Microbiol.*, 62, 329 (1970)) was purchased from the *E. coli* Genetic Stock Center, Yale University (New Haven, CT) and was transformed with *Klebsiella* cosmid DNA to give the recombinant organisms AA200-pKP1 and AA200-pKP2, containing the glycerol dehydratase gene, and AA200-pKP4, containing the diol dehydratase gene.

#### 20 DH5 $\alpha$ :

Six transformation plates containing approximately 1,000 colonies of *E. coli* XL1-Blue MR transfected with *K. pneumoniae* DNA were washed with 5 mL LB medium and centrifuged. The bacteria were pelleted and resuspended in 25 5 mL LB medium + glycerol. An aliquot (50  $\mu$ L) was inoculated into a 15 mL tube containing S12 synthetic medium with 0.2% glycerol + 400 ng per mL of vitamin B<sub>12</sub> + 0.001% yeast extract + 50amp. The tube was filled with the medium to the top and wrapped with parafilm and incubated at 30 °C. A slight turbidity was observed after 48 h. Aliquots, analyzed for product distribution as 30 described above at 78 h and 132 h, were positive for 1,3-propanediol, the later time points containing increased amounts of 1,3-propanediol.

The bacteria, testing positive for 1,3-propanediol production, were serially diluted and plated onto LB-50amp plates in order to isolate single colonies. Forty-eight single colonies were isolated and checked again for the production of 35 1,3-propanediol. Cosmid DNA was isolated from 6 independent clones and transformed into *E. coli* strain DH5 $\alpha$ . The transformants were again checked for the production of 1,3-propanediol. Two transformants were characterized further and designated as DH5 $\alpha$ -pKP1 and DH5 $\alpha$ -pKP2.

A 12.1 kb EcoRI-Sall fragment from pKP1, subcloned into pIB131 (IBI Biosystem, New Haven, CT), was sequenced and termed pHK28-26 (SEQ ID NO:19). Sequencing revealed the loci of the relevant open reading frames of the

- 5 Referring to SEQ ID NO:19, a fragment of the open reading frame for *dhaK* encoding dihydroxyacetone kinase is found at bases 1-399; the open reading frame *dhaD* encoding glycerol dehydrogenase is found at bases 983-2107; the open reading frame *dhaR* encoding the repressor is found at bases 2209-4134; the open reading frame *dhaT* encoding 1,3-propanediol oxidoreductase is found at bases
- 10 5017-6180; the open reading frame *dhaB1* encoding the alpha subunit glycerol dehydratase is found at bases 7044-8711; the open reading frame *dhaB2* encoding the beta subunit glycerol dehydratase is found at bases 8724-9308; the open reading frame *dhaB3* encoding the gamma subunit glycerol dehydratase is found at bases 9311-9736; and the open reading frame *dhaBX*, encoding a protein of
- 15 unknown function is found at bases 9749-11572.

- Single colonies of *E. coli* XL1-Blue MR transfected with packaged cosmid DNA from *K. pneumoniae* were inoculated into microtiter wells containing 200 uL of S15 medium (ammonium sulfate, 10 mM; potassium phosphate buffer, pH 7.0, 1 mM; MOPS/KOH buffer, pH 7.0, 50 mM; MgCl<sub>2</sub>, 2 mM; CaCl<sub>2</sub>,
- 20 0.7 mM; MnCl<sub>2</sub>, 50 uM; FeCl<sub>3</sub>, 1 uM; ZnCl, 1 uM; CuSO<sub>4</sub>, 1.72 uM; CoCl<sub>2</sub>, 2.53 uM; Na<sub>2</sub>MoO<sub>4</sub>, 2.42 uM; and thiamine hydrochloride, 2 uM) + 0.2% glycerol + 400 ng/mL of vitamin B<sub>12</sub> + 0.001% yeast extract + 50 ug/mL ampicillin. In addition to the microtiter wells, a master plate containing LB-50 amp was also inoculated. After 96 h, 100 uL was withdrawn and
- 25 centrifuged in a Rainin microfuge tube containing a 0.2 micron nylon membrane filter. Bacteria were retained and the filtrate was processed for HPLC analysis. Positive clones demonstrating 1,3-propanediol production were identified after screening approximately 240 colonies. Three positive clones were identified, two of which had grown on LB-50 amp and one of which had not. A single colony,
- 30 isolated from one of the two positive clones grown on LB-50 amp and verified for the production of 1,3-propanediol, was designated as pKP4. Cosmid DNA was isolated from *E. coli* strains containing pKP4 and *E. coli* strain DH5α was transformed. An independent transformant, designated as DH5α-pKP4, was verified for the production of 1,3-propanediol.

35 ECL707:

*E. coli* strain ECL707 was transformed with cosmid *K. pneumoniae* DNA corresponding to one of pKP1, pKP2, pKP4 or the Supercos vector alone and named ECL707-pKP1, ECL707-pKP2, ECL707-pKP4, and ECL707-sc,

respectively. ECL707 is defective in *glpK*, *gld*, and *ptsD* which encode the ATP-dependent glycerol kinase, NAD<sup>+</sup>-linked glycerol dehydrogenase, and enzyme II for dihydroxyacetone of the phosphoenolpyruvate-dependent phosphotransferase system, respectively.

- 5 Twenty single colonies of each cosmid transformation and five of the Supercos vector alone (negative control) transformation, isolated from LB-50 amp plates, were transferred to a master LB-50 amp plate. These isolates were also tested for their ability to convert glycerol to 1,3-propanediol in order to determine if they contained dehydratase activity. The transformants were transferred with a  
10 sterile toothpick to microtiter plates containing 200 uL of Medium A supplemented with either 0.2% glycerol or 0.2% glycerol plus 0.2% D-glucose. After incubation for 48 hr at 30 °C, the contents of the microtiter plate wells were filtered through an 0.45 micron nylon filter and chromatographed by HPLC. The results of these tests are given in Table 1.

15

Table 1  
Conversion of glycerol to 1,3-propanediol by transformed ECL707

<u>Transformant</u>	<u>Glycerol*</u>	<u>Glycerol plus Glucose*</u>
ECL707-pKP1	19/20	19/20
ECL707-pKP2	18/20	20/20
ECL707-pKP4	0/20	20/20
ECL707-sc	0/5	0/5

\*(Number of positive isolates/number of isolates tested)

#### AA200:

*E. coli* strain AA200 was transformed with cosmid *K. pneumoniae* DNA corresponding to one of pKP1, pKP2, pKP4 and the Supercos vector alone and named AA200-pKP1, AA200-pKP2, AA200-pKP4, and AA200-sc, respectively.

- 20 Strain AA200 is defective in triosephosphate isomerase (*tpi*<sup>-</sup>).

Twenty single colonies of each cosmid transformation and five of the empty vector transformation were isolated and tested for their ability to convert glycerol to 1,3-propanediol as described for *E. coli* strain ECL707. The results of these tests are given in Table 2.

Table 2  
Conversion of glycerol to 1,3-propanediol by transformed AA200

<u>Transformant</u>	<u>Glycerol*</u>	<u>Glycerol plus Glucose*</u>
AA200-pKP1	17/20	17/20
AA200-pKP2	17/20	17/20
AA200-pKP4	2/20	16/20
AA200-sc	0/5	0/5

\*(Number of positive isolates/number of isolates tested)

## EXAMPLE 2

### CONVERSION OF D-GLUCOSE TO 1,3-PROPANEDIOL BY RECOMBINANT *E. coli* USING DAR1, GPP2, *dhaB*, and *dhaT*

#### 5 Construction of general purpose expression plasmids for use in transformation of *Escherichia coli*

##### The expression vector pTacIQ

The *E. coli* expression vector, pTacIQ, contains the lacIq gene (Farabaugh, *Nature* 274, 5673 (1978)) and tac promoter (Amann et al., *Gene* 25, 167 (1983))  
10 inserted into the EcoRI of pBR322 (Sutcliffe et al., *Cold Spring Harb. Symp. Quant. Biol.* 43, 77 (1979)). A multiple cloning site and terminator sequence (SEQ ID NO:20) replaces the pBR322 sequence from EcoRI to SphI.

##### Subcloning the glycerol dehydratase genes (*dhaB1*, 2, 3)

The open reading frame for *dhaB3* gene (incorporating an EcoRI site at the  
15 5' end and a XbaI site at the 3' end) was amplified from pHK28-26 by PCR using primers (SEQ ID NOS:21 and 22). The product was subcloned into pLitmus29 (New England Biolab, Inc., Beverly, MA) to generate the plasmid pDHAB3 containing *dhaB3*.

The region containing the entire coding region for the four genes of the  
20 *dhaB* operon from pHK28-26 was cloned into pBluescriptII KS+ (Stratagene, La Jolla, CA) using the restriction enzymes KpnI and EcoRI to create the plasmid pM7.

The *dhaBX* gene was removed by digesting the plasmid pM7, which  
contains *dhaB(1,2,3,4)*, with ApaI and XbaI (deleting part of *dhaB3* and all of  
25 *dhaBX*). The resulting 5.9 kb fragment was purified and ligated with the 325-bp ApaI-XbaI fragment from plasmid pDHAB3 (restoring the *dhaB3* gene) to create pM11, which contains *dhaB(1,2,3)*.

The open reading frame for the *dhaB1* gene (incorporating a HindIII site  
and a consensus RBS ribosome binding site at the 5' end and a XbaI site at the 3'  
30 end) was amplified from pHK28-26 by PCR using primers (SEQ ID NO:23 and



SEQ ID NO:24). The product was subcloned into pLitmus28 (New England Biolab, Inc.) to generate the plasmid pDT1 containing *dhaB1*.

A NotI-XbaI fragment from pM11 containing part of the *dhaB1* gene, the *dhaB2* gene and the *dhaB3* gene was inserted into pDT1 to create the *dhaB* expression plasmid, pDT2. The HindIII-XbaI fragment containing the *dhaB(1,2,3)* genes from pDT2 was inserted into pTacIQ to create pDT3.

Subcloning the 1,3-propanediol dehydrogenase gene (*dhaT*)

The KpnI-SacI fragment of pHK28-26, containing the complete 1,3-propanediol dehydrogenase (*dhaT*) gene, was subcloned into pBluescriptII KS+ creating plasmid pAH1. The *dhaT* gene (incorporating an XbaI site at the 5' end and a BamHI site at the 3' end) was amplified by PCR from pAH1 as template DNA using synthetic primers (SEQ ID NO:25 with SEQ ID NO:26). The product was subcloned into pCR-Script (Stratagene) at the SrfI site to generate the plasmids pAH4 and pAH5 containing *dhaT*. The plasmid pAH4 contains the *dhaT* gene in the correct orientation for expression from the lac promoter in pCR-Script and pAH5 contains the *dhaT* gene in the opposite orientation. The XbaI-BamHI fragment from pAH4 containing the *dhaT* gene was inserted into pTacIQ to generate plasmid pAH8. The HindIII-BamHI fragment from pAH8 containing the RBS and *dhaT* gene was inserted into pBluescriptII KS+ to create pAH11. The HindIII-SalI fragment from pAH8 containing the RBS, *dhaT* gene and terminator was inserted into pBluescriptII SK+ to create pAH12.

Construction of an expression cassette for *dhaB(1,2,3)* and *dhaT*

An expression cassette for the *dhaB(1,2,3)* and *dhaT* was assembled from the individual *dhaB(1,2,3)* and *dhaT* subclones described above using standard molecular biology methods. The SpeI-KpnI fragment from pAH8 containing the RBS, *dhaT* gene and terminator was inserted into the XbaI-KpnI sites of pDT3 to create pAH23. The SmaI-EcoRI fragment between the *dhaB3* and *dhaT* gene of pAH23 was removed to create pAH26. The SpeI-NotI fragment containing an EcoRI site from pDT2 was used to replace the SpeI-NotI fragment of pAH26 to generate pAH27.

Construction of expression cassette for *dhaT* and *dhaB(1,2,3)*

An expression cassette for *dhaT* and *dhaB(1,2,3)* was assembled from the individual *dhaB(1,2,3)* and *dhaT* subclones described previously using standard molecular biology methods. A SpeI-SacI fragment containing the *dhaB(1,2,3)* genes from pDT3 was inserted into pAH11 at the SpeI-SacI sites to create pAH24.

Cloning and expression of glycerol 3-phosphatase for increased glycerol production in *E. coli*

The *Saccharomyces cerevisiae* chromosome V lamda clone 6592 (Gene Bank, accession # U18813x11) was obtained from ATCC. The glycerol 3-phosphate phosphatase (GPP2) gene (incorporating an BamHI-RBS-XbaI site at the 5' end and a SmaI site at the 3' end) was cloned by PCR cloning from the lamda clone as target DNA using synthetic primers (SEQ ID NO:27 with SEQ ID NO:28). The product was subcloned into pCR-Script (Stratagene) at the SrfI site to generate the plasmids pAH15 containing GPP2. The plasmid pAH15 contains the GPP2 gene in the inactive orientation for expression from the lac promoter in pCR-Script SK+. The BamHI-SmaI fragment from pAH15 containing the GPP2 gene was inserted into pBlueScriptII SK+ to generate plasmid pAH19. The pAH19 contains the GPP2 gene in the correct orientation for expression from the lac promoter. The XbaI-PstI fragment from pAH19 containing the GPP2 gene was inserted into pPHOX2 to create plasmid pAH21.

Plasmids for the expression of *dhaT*, *dhaB*(1,2,3) and GPP2 genes

A Sall-EcoRI-XbaI linker (SEQ ID NOS:29 and 30) was inserted into pAH5 which was digested with the restriction enzymes, Sall-XbaI to create pDT16. The linker destroys the XbaI site. The 1 kb Sall-MluI fragment from pDT16 was then inserted into pAH24 replacing the existing Sall-MluI fragment to create pDT18.

The 4.1 kb EcoRI-XbaI fragment containing the expression cassette for *dhaT* and *dhaB*(1,2,3) from pDT18 and the 1.0 kb XbaI-Sall fragment containing the GPP2 gene from pAH21 was inserted into the vector pMMB66EH (Füste et al., *GENE*, 48, 119 (1986)) digested with the restriction enzymes EcoRI and Sall to create pDT20.

Plasmids for the over-expression of DAR1 in *E. coli*

DAR1 was isolated by PCR cloning from genomic *S. cerevisiae* DNA using synthetic primers (SEQ ID NO:46 with SEQ ID NO:47). Successful PCR cloning places an NcoI site at the 5' end of DAR1 where the ATG within NcoI is the DAR1 initiator methionine. At the 3' end of DAR1 a BamHI site is introduced following the translation terminator. The PCR fragments were digested with NcoI + BamHI and cloned into the same sites within the expression plasmid pTrc99A (Pharmacia, Piscataway, New Jersey) to give pDAR1A.

In order to create a better ribosome binding site at the 5' end of DAR1, a SpeI-RBS-NcoI linker obtained by annealing synthetic primers (SEQ ID NO:48 with SEQ ID NO:49) was inserted into the NcoI site of pDAR1A to create pAH40. Plasmid pAH40 contains the new RBS and DAR1 gene in the correct

orientation for expression from the trc promoter of Trc99A (Pharmacia). The NcoI-BamHI fragment from pDAR1A and a second set of SpeI-RBS-NcoI linker obtained by annealing synthetic primers (SEQ ID NO:31 with SEQ ID NO:32) was inserted into the SpeI-BamHI site of pBluescript II-SK+ (Stratagene) to create pAH41. The construct pAH41 contains an ampicillin resistance gene. The NcoI-BamHI fragment from pDAR1A and a second set of SpeI-RBS-NcoI linker obtained by annealing synthetic primers (SEQ ID NO:31 with SEQ ID NO:32) was inserted into the SpeI-BamHI site of pBC-SK+ (Stratagene) to create pAH42. The construct pAH42 contains a chloramphenicol resistance gene.

10 Construction of an expression cassette for DAR1 and GPP2

An expression cassette for DAR1 and GPP2 was assembled from the individual DAR1 and GPP2 subclones described above using standard molecular biology methods. The BamHI-PstI fragment from pAH19 containing the RBS and GPP2 gene was inserted into pAH40 to create pAH43. The BamHI-PstI fragment from pAH19 containing the RBS and GPP2 gene was inserted into pAH41 to create pAH44. The same BamHI-PstI fragment from pAH19 containing the RBS and GPP2 gene was also inserted into pAH42 to create pAH45.

E. coli strain construction

20 *E. coli* W1485 is a wild-type K-12 strain (ATCC 12435). This strain was transformed with the plasmids pDT20 and pAH42 and selected on LA (Luria Agar, Difco) plates supplemented with 50 µg/mL carbencillim and 10 µg/mL chloramphenicol.

Production of 1,3-propanediol from glucose

25 *E. coli* W1485/pDT20/pAH42 was transferred from a plate to 50 mL of a medium containing per liter: 22.5 g glucose, 6.85 g K<sub>2</sub>HPO<sub>4</sub>, 6.3 g (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 0.5 g NaHCO<sub>3</sub>, 2.5 g NaCl, 8 g yeast extract, 8 g tryptone, 2.5 mg vitamin B<sub>12</sub>, 2.5 mL modified Balch's trace-element solution, 50 mg carbencillim and 10 mg chloramphenicol, final pH 6.8 (HCl), then filter sterilized. The composition of modified Balch's trace-element solution can be found in Methods for General and  
30 Molecular Bacteriology (P. Gerhardt et al., eds, p. 158, American Society for Microbiology, Washington, DC (1994)). After incubating at 37 °C, 300 rpm for 6 h, 0.5 g glucose and IPTG (final concentration = 0.2 mM) were added and shaking was reduced to 100 rpm. Samples were analyzed by GC/MS. After 24 h,  
35 W1485/pDT20/pAH42 produced 1.1 g/L glycerol and 195 mg/L 1,3-propanediol.

EXAMPLE 3  
CLONING AND EXPRESSION OF *dhaB* AND *dhaT*  
IN *Saccharomyces cerevisiae*

Expression plasmids that could exist as replicating episomal elements were constructed for each of the four *dha* genes. For all expression plasmids a yeast ADH1 promoter was present and separated from a yeast ADH1 transcription terminator by fragments of DNA containing recognition sites for one or more restriction endonucleases. Each expression plasmid also contained the gene for  $\beta$ -lactamase for selection in *E. coli* on media containing ampicillin, an origin of replication for plasmid maintenance in *E. coli*, and a 2 micron origin of replication for maintenance in *S. cerevisiae*. The selectable nutritional markers used for yeast and present on the expression plasmids were one of the following: HIS3 gene encoding imidazoleglycerolphosphate dehydratase, URA3 gene encoding orotidine 5'-phosphate decarboxylase, TRP1 gene encoding N-(5'-phosphoribosyl)-anthranilate isomerase, and LEU2 encoding  $\beta$ -isopropylmalate dehydrogenase.

The open reading frames for *dhaT*, *dhaB3*, *dhaB2* and *dhaB1* were amplified from pHK28-26 (SEQ ID NO:19) by PCR using primers (SEQ ID NO:38 with SEQ ID NO:39, SEQ ID NO:40 with SEQ ID NO:41, SEQ ID NO:42 with SEQ ID NO:43, and SEQ ID NO:44 with SEQ ID NO:45 for *dhaT*, *dhaB3*, *dhaB2* and *dhaB1*, respectively) incorporating EcoR1 sites at the 5' ends (10 mM Tris pH 8.3, 50 mM KCl, 1.5 mM MgCl<sub>2</sub>, 0.0001% gelatin, 200  $\mu$ M dATP, 200  $\mu$ M dCTP, 200  $\mu$ M dGTP, 200  $\mu$ M dTTP, 1  $\mu$ M each primer, 1-10 ng target DNA, 25 units/mL Amplitaq™ DNA polymerase (Perkin-Elmer Cetus, Norwalk CT)). PCR parameters were 1 min at 94 °C, 1 min at 55 °C, 1 min at 72 °C, 35 cycles. The products were subcloned into the EcoR1 site of pHIL-D4 (Phillips Petroleum, Bartlesville, OK) to generate the plasmids pMP13, pMP14, pMP20 and pMP15 containing *dhaT*, *dhaB3*, *dhaB2* and *dhaB1*, respectively.

Construction of *dhaB1* expression plasmid pMCK10

The 7.8 kb replicating plasmid pGADGH (Clontech, Palo Alto, CA) was digested with HindIII, dephosphorylated, and ligated to the *dhaB1* HindIII fragment from pMP15. The resulting plasmid (pMCK10) had *dhaB1* correctly oriented for transcription from the ADH1 promoter and contained a LEU2 marker.

Construction of *dhaB2* expression plasmid pMCK17

Plasmid pGADGH (Clontech, Palo Alto, CA) was digested with HindIII and the single-strand ends converted to EcoRI ends by ligation with HindIII-XmnI and EcoRI-XmnI adaptors (New England Biolabs, Beverly, MA). Selection for plasmids with correct EcoRI ends was achieved by ligation to a kanamycin

resistance gene on an EcoRI fragment from plasmid pUC4K (Pharmacia Biotech, Uppsala), transformation into *E. coli* strain DH5 $\alpha$  and selection on LB plates containing 25  $\mu$ g/mL kanamycin. The resulting plasmid (pGAD/KAN2) was digested with SnaBI and EcoRI and a 1.8 kb fragment with the ADH1 promoter was isolated. Plasmid pGBT9 (Clontech, Palo Alto, CA) was digested with SnaBI and EcoRI, and the 1.5 kb ADH1/GAL4 fragment replaced by the 1.8 kb ADH1 promoter fragment isolated from pGAD/KAN2 by digestion with SnaBI and EcoRI. The resulting vector (pMCK11) is a replicating plasmid in yeast with an ADH1 promoter and terminator and a TRP1 marker. Plasmid pMCK11 was digested with EcoRI, dephosphorylated, and ligated to the *dhaB2* EcoRI fragment from pMP20. The resulting plasmid (pMCK17) had *dhaB2* correctly oriented for transcription from the ADH1 promoter and contained a TRP1 marker.

#### Construction of *dhaB3* expression plasmid pMCK30

Plasmid pGBT9 (Clontech) was digested with NaeI and PvuII and the 1 kb TRP1 gene removed from this vector. The TRP1 gene was replaced by a URA3 gene donated as a 1.7 kb AatII/NaeI fragment from plasmid pRS406 (Stratagene) to give the intermediary vector pMCK32. The truncated ADH1 promoter present on pMCK32 was removed on a 1.5 kb SnaBI/EcoRI fragment, and replaced with a full-length ADH1 promoter on a 1.8 kb SnaBI/EcoRI fragment from plasmid pGAD/KAN2 to yield the vector pMCK26. The unique EcoRI site on pMCK26 was used to insert an EcoRI fragment with *dhaB3* from plasmid pMP14 to yield pMCK30. The pMCK30 replicating expression plasmid has *dhaB3* orientated for expression from the ADH1 promoter, and has a URA3 marker.

#### Construction of *dhaT* expression plasmid pMCK35

Plasmid pGBT9 (Clontech) was digested with NaeI and PvuII and the 1 kb TRP1 gene removed from this vector. The TRP1 gene was replaced by a HIS3 gene donated as an XmnI/NaeI fragment from plasmid pRS403 (Stratagene) to give the intermediary vector pMCK33. The truncated ADH1 promoter present on pMCK33 was removed on a 1.5 kb SnaBI/EcoRI fragment, and replaced with a full-length ADH1 promoter on a 1.8 kb SnaBI/EcoRI fragment from plasmid pGAD/KAN2 to yield the vector pMCK31. The unique EcoRI site on pMCK31 was used to insert an EcoRI fragment with *dhaT* from plasmid pMP13 to yield pMCK35. The pMCK35 replicating expression plasmid has *dhaT* orientated for expression from the ADH1 promoter, and has a HIS3 marker.

#### Transformation of *S. cerevisiae* with *dha* expression plasmids

*S. cerevisiae* strain YPH500 (*ura3-52 lys2-801 ade2-101 trp1- $\Delta$ 63 his3- $\Delta$ 200 leu2- $\Delta$ 1*) (Sikorski R. S. and Hieter P., *Genetics* 122, 19-27, (1989)) purchased from Stratagene (La Jolla, CA) was transformed with 1-2  $\mu$ g of plasmid

DNA using a Frozen-EZ Yeast Transformation Kit (Catalog #T2001) (Zymo Research, Orange, CA). Colonies were grown on Supplemented Minimal Medium (SMM - 0.67% yeast nitrogen base without amino acids, 2% glucose) for 3-4 d at 29 °C with one or more of the following additions: adenine sulfate (20 mg/L), uracil (20 mg/L), L-tryptophan (20 mg/L), L-histidine (20 mg/L), L-leucine (30 mg/L), L-lysine (30 mg/L). Colonies were streaked on selective plates and used to inoculate liquid media.

Screening of *S. cerevisiae* transformants for *dha* genes

Chromosomal DNA from URA<sup>+</sup>, HIS<sup>+</sup>, TRP<sup>+</sup>, LEU<sup>+</sup> transformants was analyzed by PCR using primers specific for each gene (SEQ ID NOS:38-45). The presence of all four open reading frames was confirmed.

Expression of *dhaB* and *dhaT* activity in transformed *S. cerevisiae*

The presence of active glycerol dehydratase (*dhaB*) and 1,3-propanediol oxido-reductase (*dhaT*) was demonstrated using *in vitro* enzyme assays. Additionally, western blot analysis confirmed protein expression from all four open reading frames.

Strain YPH500, transformed with the group of plasmids pMCK10, pMCK17, pMCK30 and pMCK35, was grown on Supplemented Minimal Medium containing 0.67% yeast nitrogen base without amino acids 2% glucose 20 mg/L adenine sulfate, and 30 mg/L L-lysine. Cells were homogenized and extracts assayed for *dhaB* activity. A specific activity of 0.12 units per mg protein was obtained for glycerol dehydratase, and 0.024 units per mg protein for 1,3-propanediol oxido-reductase.

EXAMPLE 4

PRODUCTION OF 1,3-PROPANEDIOL FROM D-GLUCOSE  
USING RECOMBINANT *Saccharomyces cerevisiae*

*S. cerevisiae* YPH500, harboring the groups of plasmids pMCK10, pMCK17, pMCK30 and pMCK35, was grown in a BiostatB fermenter (B Braun Biotech, Inc.) in 1.0 L of minimal medium initially containing 20 g/L glucose, 6.7 g/L yeast nitrogen base without amino acids, 40 mg/L adenine sulfate and 60 mg/L L-lysine·HCl. During the course of the growth, an additional equivalent of yeast nitrogen base, adenine and lysine was added. The fermenter was controlled at pH 5.5 with addition of 10% phosphoric acid and 2 M NaOH, 30 °C, and 40% dissolved oxygen tension through agitation control. After 38 h, the cells (OD<sub>600</sub> = 5.8 AU) were harvested by centrifugation and resuspended in base medium (6.7 g/L yeast nitrogen base without amino acids, 20 mg/L adenine sulfate, 30 mg/L L-lysine·HCl, and 50 mM potassium phosphate buffer, pH 7.0).

Reaction mixtures containing cells ( $OD_{600} = 20$  AU) in a total volume of 4 mL of base media supplemented with 0.5% glucose, 5 ug/mL coenzyme B<sub>12</sub> and 0, 10, 20, or 40 mM chloroquine were prepared, in the absence of light and oxygen (nitrogen sparging), in 10 mL crimp sealed serum bottles and incubated at 30 °C with shaking. After 30 h, aliquots were withdrawn and analyzed by HPLC. The results are shown in the Table 3.

**Table 3**  
Production of 1,3-propanediol using recombinant *S. cerevisiae*

reaction	chloroquine (mM)	1,3-propanediol (mM)
1	0	0.2
2	10	0.2
3	20	0.3
4	40	0.7

#### EXAMPLE 5

#### 10 USE OF A *S. cerevisiae* DOUBLE TRANSFORMANT FOR PRODUCTION OF 1,3-PROPANEDIOL FROM D-GLUCOSE WHERE *dhaB* AND *dhaT* ARE INTEGRATED INTO THE GENOME

Example 5 prophetically demonstrates the transformation of *S. cerevisiae* with *dhaB1*, *dhaB2*, *dhaB3*, and *dhaT* and the stable integration of the genes into the yeast genome for the production of 1,3-propanediol from glucose.

##### Construction of expression cassettes

Four expression cassettes (*dhaB1*, *dhaB2*, *dhaB3*, and *dhaT*) are constructed for glucose-induced and high-level constitutive expression of these genes in yeast, *Saccharomyces cerevisiae*. These cassettes consist of: (i) the phosphoglycerate kinase (PGK) promoter from *S. cerevisiae* strain S288C; (ii) one of the genes *dhaB1*, *dhaB2*, *dhaB3*, or *dhaT*; and (iii) the PGK terminator from *S. cerevisiae* strain S288C. The PCR-based technique of gene splicing by overlap extension (Horton et al., *BioTechniques*, 8:528-535, (1990)) is used to recombine DNA sequences to generate these cassettes with seamless joints for optimal expression of each gene. These cassettes are cloned individually into a suitable vector (pLITMUS 39) with restriction sites amenable to multi-cassette cloning in yeast expression plasmids.

##### Construction of yeast integration vectors

Vectors used to effect the integration of expression cassettes into the yeast genome are constructed. These vectors contain the following elements: (i) a polycloning region into which expression cassettes are subcloned; (ii) a unique marker used to select for stable yeast transformants; (iii) replication origin and

selectable marker allowing gene manipulation in *E. coli* prior to transforming yeast. One integration vector contains the *URA3* auxotrophic marker (YIp352b), and a second integration vector contains the *LYS2* auxotrophic marker (pKP7).

#### Construction of yeast expression plasmids

- 5           Expression cassettes for *dhaB1* and *dhaB2* are subcloned into the polycloning region of the YIp352b (expression plasmid #1), and expression cassettes for *dhaB3* and *dhaT* are subcloned into the polycloning region of pKP7 (expression plasmid #2).

#### Transformation of yeast with expression plasmids

- 10           *S. cerevisiae* (*ura3*, *lys2*) is transformed with expression plasmid #1 using Frozen-EZ Yeast Transformation kit (Zymo Research, Orange, CA), and transformants selected on plates lacking uracil. Integration of expression cassettes for *dhaB1* and *dhaB2* is confirmed by PCR analysis of chromosomal DNA. Selected transformants are re-transformed with expression plasmid #2 using
- 15           Frozen-EZ Yeast Transformation kit, and double transformants selected on plates lacking lysine. Integration of expression cassettes for *dhaB3* and *dhaT* is confirmed by PCR analysis of chromosomal DNA. The presence of all four expression cassettes (*dhaB1*, *dhaB2*, *dhaB3*, *dhaT*) in double transformants is confirmed by PCR analysis of chromosomal DNA.

- 20           Protein production from double-transformed yeast

Production of proteins encoded by *dhaB1*, *dhaB2*, *dhaB3* and *dhaT* from double-transformed yeast is confirmed by Western blot analysis.

#### Enzyme activity from double-transformed yeast

- 25           Active glycerol dehydratase and active 1,3-propanediol dehydrogenase from double-transformed yeast is confirmed by enzyme assay as described in General Methods above.

#### Production of 1,3-propanediol from double-transformed yeast

Production of 1,3-propanediol from glucose in double-transformed yeast is demonstrated essentially as described in Example 4.

- 30           EXAMPLE 6

#### CONSTRUCTION OF PLASMIDS CONTAINING DARI/GPP2

#### OR *dhaT/dhaB1-3* AND TRANSFORMATION INTO *KLEBSIELLA* SPECIES

- K. pneumoniae* (ATCC 25955), *K. pneumoniae* (ECL2106), and *K. oxytoca* (ATCC 8724) are naturally resistant to ampicillin (up to 150 ug/mL)
- 35           and kanamycin (up to 50 ug/mL), but sensitive to tetracycline (10 ug/mL) and chloramphenicol (25 ug/mL). Consequently, replicating plasmids which encode resistance to these latter two antibiotics are potentially useful as cloning vectors for these *Klebsiella* strains. The wild-type *K. pneumoniae* (ATCC 25955), the



glucose-derepressed *K. pneumonia* (ECL2106), and *K. oxytoca* (ATCC 8724) were successfully transformed to tetracycline resistance by electroporation with the moderate-copy-number plasmid, pBR322 (New England Biolabs, Beverly, MA). This was accomplished by the following procedure: Ten mL of an  
5 overnight culture was inoculated into 1 L LB (1% (w/v) Bacto-tryptone (Difco, Detroit, MI), 0.5% (w/v) Bacto-yeast extract (Difco) and 0.5% (w/v) NaCl (Sigma, St. Louis, MO) and the culture was incubated at 37 °C to an OD<sub>600</sub> of 0.5-0.7. The cells were chilled on ice, harvested by centrifugation at 4000 x g for 15 min, and resuspended in 1 L ice-cold sterile 10% glycerol. The cells were  
10 repeatedly harvested by centrifugation and progressively resuspended in 500 mL, 20 mL and, finally, 2 mL ice-cold sterile 10% glycerol. For electroporation, 40 uL of cells were mixed with 1-2 uL DNA in a chilled 0.2 cm cuvette and were pulsed at 200 Ω, 2.5 kV for 4-5 msec using a BioRad Gene Pulser (BioRad, Richmond, CA). One μL of SOC medium (2% (w/v) Bacto-tryptone (Difco),  
15 0.5% (w/v) Bacto-yeast extract (Difco), 10 μM NaCl, 10 μM MgCl<sub>2</sub>, 10 μM MgSO<sub>4</sub>, 2.5 μM KCl and 20 μM glucose) was added to the cells and, after the suspension was transferred to a 17 x 100 mm sterile polypropylene tube, the culture was incubated for 1 hr at 37 °C, 225 rpm. Aliquots were plated on selective medium, as indicated. Analyses of the plasmid DNA from independent  
20 tetracycline-resistant transformants showed the restriction endonuclease digestion patterns typical of pBR322, indicating that the vector was stably maintained after overnight culture at 37 °C in LB containing tetracycline (10 ug/mL). Thus, this vector, and derivatives such as pBR329 (ATCC 37264) which encodes resistance to ampicillin, tetracycline and chloramphenicol, may be used to introduce the  
25 *DAR1/GPP2* and *dhaT/dhaB1-3* expression cassettes into *K. pneumoniae* and *K. oxytoca*.

The *DAR1* and *GPP2* genes may be obtained by PCR-mediated amplification from the *Saccharomyces cerevisiae* genome, based on their known DNA sequence. The genes are then transformed into *K. pneumoniae* or *K. oxytoca*  
30 under the control of one or more promoters that may be used to direct their expression in media containing glucose. For convenience, the genes were obtained on a 2.4 kb DNA fragment obtained by digestion of plasmid pAH44 with the *PvuII* restriction endonuclease, whereby the genes are already arranged in an expression cassette under the control of the *E. coli lac* promoter. This DNA  
35 fragment was ligated to *PvuII*-digested pBR329, producing the insertional inactivation of its chloramphenicol resistance gene. The ligated DNA was used to transform *E. coli* DH5α (Gibco, Gaithersburg, MD). Transformants were selected by their resistance to tetracycline (10 ug/mL) and were screened for their

sensitivity to chloramphenicol (25 ug/mL). Analysis of the plasmid DNA from tetracycline-resistant, chloramphenicol-sensitive transformants confirmed the presence of the expected plasmids, in which the P<sub>lac</sub>-*dar1-gpp2* expression cassette was subcloned in either orientation into the pBR329 PvuII site. These  
5 plasmids, designated pJSP1A (clockwise orientation) and pJSP1B (counter-clockwise orientation), were separately transformed by electroporation into *K. pneumonia* (ATCC 25955), *K. pneumonia* (ECL2106) and *K. oxytoca* (ATCC 8724) as described. Transformants were selected by their resistance to tetracycline (10 ug/mL) and were screened for their sensitivity to chloramphenicol  
10 (25 ug/mL). Restriction analysis of the plasmids isolated from independent transformants showed only the expected digestion patterns, and confirmed that they were stably maintained at 37 °C with antibiotic selection. The expression of the *DAR1* and *GPP2* genes may be enhanced by the addition of IPTG (0.2-2.0 mM) to the growth medium.

15 The four *K. pneumoniae* *dhaB*(1-3) and *dhaT* genes may be obtained by PCR-mediated amplification from the *K. pneumoniae* genome, based on their known DNA sequence. These genes are then transformed into *K. pneumoniae* under the control of one or more promoters that may be used to direct their expression in media containing glucose. For convenience, the genes were  
20 obtained on an approximately 4.0 kb DNA fragment obtained by digestion of plasmid pAH24 with the *KpnI/SacI* restriction endonucleases, whereby the genes are already arranged in an expression cassette under the control of the *E. coli lac* promoter. This DNA fragment was ligated to similarly digested pBC-KS+ (Stratagene, LaJolla, CA) and used to transform *E. coli* DH5α. Transformants  
25 were selected by their resistance to chloramphenicol (25 ug/mL) and were screened for a white colony phenotype on LB agar containing X-gal. Restriction analysis of the plasmid DNA from chloramphenicol-resistant transformants demonstrating the white colony phenotype confirmed the presence of the expected plasmid, designated pJSP2, in which the *dhaT-dhaB*(1-3) genes were subcloned  
30 under the control of the *E. coli lac* promoter.

To enhance the conversion of glucose to 3G, this plasmid was separately transformed by electroporation into *K. pneumoniae* (ATCC 25955) (pJSP1A), *K. pneumoniae* (ECL2106) (pJSP1A) and *K. oxytoca* (ATCC 8724) (pJSP1A) already containing the P<sub>lac</sub>-*dar1-gpp2* expression cassette. Cotransformants were  
35 selected by their resistance to both tetracycline (10 ug/mL) and chloramphenicol (25 ug/mL). Restriction analysis of the plasmids isolated from independent cotransformants showed the digestion patterns expected for both pJSP1A and

pJSP2. The expression of the *DAR1*, *GPP2*, *dhaB(1-3)*, and *dhaT* genes may be enhanced by the addition of IPTG (0.2-2.0 mM) to the medium.

#### EXAMPLE 7

##### Production of 1,3 propanediol from glucose by *K. pneumoniae*

5 *Klebsiella pneumoniae* strains ECL 2106 and 2106-47, both transformed with pJSP1A, and ATCC 25955, transformed with pJSP1A and pJSP2, were grown in a 5 L Applikon fermenter under various conditions (see Table 4) for the production of 1,3-propanediol from glucose. Strain 2104-47 is a fluoroacetate-tolerant derivative of ECL 2106 which was obtained from a fluoroacetate/lactate selection plate as described in Bauer et al., *Appl. Environ. Microbiol.* 56, 1296  
10 (1990). In each case, the medium used contained 50-100 mM potassium phosphate buffer, pH 7.5, 40 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 0.1% (w/v) yeast extract, 10 μM CoCl<sub>2</sub>, 6.5 μM CuCl<sub>2</sub>, 100 μM FeCl<sub>3</sub>, 18 μM FeSO<sub>4</sub>, 5 μM H<sub>3</sub>BO<sub>3</sub>, 50 μM MnCl<sub>2</sub>, 0.1 μM Na<sub>2</sub>MoO<sub>4</sub>, 25 μM ZnCl<sub>2</sub>, 0.82 mM MgSO<sub>4</sub>, 0.9 mM CaCl<sub>2</sub>, and 10-20 g/L  
15 glucose. Additional glucose was fed, with residual glucose maintained in excess. Temperature was controlled at 37 °C and pH controlled at 7.5 with 5N KOH or NaOH. Appropriate antibiotics were included for plasmid maintenance; IPTG (isopropyl-β-D-thiogalactopyranoside) was added at the indicated concentrations as well. For anaerobic fermentations, 0.1 vvm nitrogen was sparged through the reactor; when the dO setpoint was 5%, 1 vvm air was sparged through the reactor  
20 and the medium was supplemented with vitamin B12. Final concentrations and overall yields (g/g) are shown in Table 4.

Table 4

Production of 1,3 propanediol from glucose by *K. pneumoniae*

Organism	dO	IPTG, mM	vitamin B12, mg/L	Titer, g/L	Yield, g/g
25955[pJSP1A/pJSP2]	0	0.5	0	8.1	16%
25955[pJSP1A/pJSP2]	5%	0.2	0.5	5.2	4%
2106[pJSP1A]	0	0	0	4.9	17%
2106[pJSP1A]	5%	0	5	6.5	12%
2106-47[pJSP1A]	5%	0.2	0.5	10.9	12%

**EXAMPLE 8****Conversion of carbon substrates to 1,3-propanediol by recombinant*****K. pneumoniae* containing *dar1*, *gpp2*, *dhaB*, and *dhaT*****A. Conversion of D-fructose to 1,3-propanediol by various *K. pneumoniae***

5 recombinant strains:

Single colonies of *K. pneumoniae* (ATCC 25955 pJSP1A), *K. pneumoniae* (ATCC 25955 pJSP1A/pJSP2), *K. pneumoniae* (ATCC 2106 pJSP1A), and *K. pneumoniae* (ATCC 2106 pJSP1A/pJSP2) were transferred from agar plates and in separate culture tubes were subcultured overnight in Luria-Bertani (LB) broth containing the appropriate antibiotic agent(s). A 50-mL flask containing 45 mL of a steri-filtered minimal medium defined as LLMM/F which contains per liter: 10 g fructose; 1 g yeast extract; 50 mmoles potassium phosphate, pH 7.5; 40 mmoles (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>; 0.09 mmoles calcium chloride; 2.38 mg CoCl<sub>2</sub>•6H<sub>2</sub>O; 0.88 mg CuCl<sub>2</sub>•2H<sub>2</sub>O; 27 mg FeCl<sub>3</sub>•6H<sub>2</sub>O; 5 mg FeSO<sub>4</sub>•7H<sub>2</sub>O; 0.31 mg H<sub>3</sub>BO<sub>3</sub>; 10 mg MnCl<sub>2</sub>•4H<sub>2</sub>O; 0.023 mg Na<sub>2</sub>MoO<sub>4</sub>•2H<sub>2</sub>O; 3.4 mg ZnCl<sub>2</sub>; 0.2 g MgSO<sub>4</sub>•7H<sub>2</sub>O. Tetracycline at 10 ug/mL was added to medium for reactions using either of the single plasmid recombinants; 10 ug/mL tetracycline and 25 ug/mL chloramphenicol for reactions using either of the double plasmid recombinants. The medium was thoroughly sparged with nitrogen prior to inoculation with 2 mL of the subculture. IPTG (I) at final concentration of 0.5 mM was added to some flasks. The flasks were capped, then incubated at 37 °C, 100 rpm in a New Brunswick Series 25 incubator/shaker. Reactions were run for at least 24 hours or until most of the carbon substrate was converted into products. Samples were analyzed by HPLC. Table 5 describes the yields of 1,3-propanediol produced from fructose by the various *Klebsiella* recombinants.

**Table 5****Production of 1,3-propanediol from D-fructose using recombinant *Klebsiella***

Klebsiella Strain	Medium	Conversion	[3G] (g/L)	Yield Carbon (%)
2106 pBR329	LLMM/F	100	0	0
2106 pJSP1A	LLMM/F	50	0.66	15.5
2106 pJSP1A	LLMM/F + I	100	0.11	1.4
2106 pJSP1A/pJSP2	LLMM/F	58	0.26	5
25955 pBR329	LLMM/F	100	0	0
25955 pJSP1A	LLMM/F	100	0.3	4
25955 pJSP1A	LLMM/F + I	100	0.15	2
25955 pJSP1A/pJSP2	LLMM/F	100	0.9	11
25955 pJSP1A/pJSP2	LLMM/F + I	62	1.0	20

B. Conversion of various carbon substrates to 1,3-propanediol by *K. pneumoniae* (ATCC 25955 pJSP1A/pJSP2):

An aliquot (0.1 mL) of frozen stock cultures of *K. pneumoniae*

- 5 (ATCC 25955 pJSP1A/pJSP2) was transferred to 50 mL Seed medium in a 250 mL baffled flask. The Seed medium contained per liter: 0.1 molar NaK/PO<sub>4</sub> buffer, pH 7.0; 3 g (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>; 5 g glucose, 0.15 g MgSO<sub>4</sub>•7H<sub>2</sub>O, 10 mL 100X Trace Element solution, 25 mg chloramphenicol, 10 mg tetracycline, and 1 g yeast extract. The 100X Trace Element contained per liter: 10 g citric acid, 1.5 g
- 10 CaCl<sub>2</sub>•2H<sub>2</sub>O, 2.8 g FeSO<sub>4</sub>•7H<sub>2</sub>O, 0.39 g ZnSO<sub>4</sub>•7H<sub>2</sub>O, 0.38 g CuSO<sub>4</sub>•5H<sub>2</sub>O, 0.2 g CoCl<sub>2</sub>•6H<sub>2</sub>O, and 0.3 g MnCl<sub>2</sub>•4H<sub>2</sub>O. The resulting solution was titrated to pH 7.0 with either KOH or H<sub>2</sub>SO<sub>4</sub>. The glucose, trace elements, antibiotics and yeast extracts were sterilized separately. The seed inoculum was grown overnight at 35 °C and 250 rpm.
- 15 The reaction design was semi-aerobic. The system consisted of 130 mL Reaction medium in 125 mL sealed flasks that were left partially open with aluminum foil strip. The Reaction Medium contained per liter: 3 g (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>; 20 g carbon substrate; 0.15 molar NaK/PO<sub>4</sub> buffer, pH 7.5; 1 g yeast extract; 0.15 g MgSO<sub>4</sub>•7H<sub>2</sub>O; 0.5 mmoles IPTG; 10 mL 100X Trace Element solution;
- 20 25 mg chloramphenicol; and 10 mg tetracycline. The resulting solution was titrated to pH 7.5 with KOH or H<sub>2</sub>SO<sub>4</sub>. The carbon sources were: D-glucose (Glc); D-fructose (Frc); D-lactose (Lac); D-sucrose (Suc); D-maltose (Mal); and D-mannitol (Man). A few glass beads were included in the medium to improve mixing. The reactions were initiated by addition of seed inoculum so that the
- 25 optical density of the cell suspension started at 0.1 AU as measured at λ<sub>600</sub> nm. The flasks were incubated at 35 °C: 250 rpm. 3G production was measured by HPLC after 24 hr. Table 6 describes the yields of 1,3-propanediol produced from the various carbon substrates.

30

**Table 6**  
Production of 1,3-propanediol from various carbon substrates  
using recombinant *Klebsiella* 25955 pJSP1A/pJSP2

Carbon Substrate	1,3-Propanediol (g/L)		
	Expt. 1	Expt. 2	Expt 3
Glc	0.89	1	1.6
Frc	0.19	0.23	0.24
Lac	0.15	0.58	0.56
Suc	0.88	0.62	
Mal	0.05	0.03	0.02
Man	0.03	0.05	0.04

SEQUENCE LISTING

## (1) GENERAL INFORMATION:

## (i) APPLICANT:

(A) ADDRESSEE: E. I. DU PONT DE NEMOURS AND COMPANY  
(B) STREET: 1007 MARKET STREET  
(C) CITY: WILMINGTON  
(D) STATE: DELAWARE  
(E) COUNTRY: U.S.A.  
(F) ZIP: 19898  
(G) TELEPHONE: 302-892-8112  
(H) TELEFAX: 302-773-0164  
(I) TELEX: 6717325

(A) ADDRESSEE: GENENCOR INTERNATIONAL, INC.  
(B) STREET: 4 CAMBRIDGE PLACE  
1870 SOUTH WINTON ROAD  
(C) CITY: ROCHESTER  
(D) STATE: NEW YORK  
(E) COUNTRY: U.S.A.  
(F) POSTAL CODE (ZIP): 14618

(ii) TITLE OF INVENTION: METHOD FOR THE RECOMBINANT  
PRODUCTION OF 1,3-PROPANEDIOL

(iii) NUMBER OF SEQUENCES: 49

## (iv) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: 3.50 INCH DISKETTE  
(B) COMPUTER: IBM PC COMPATIBLE  
(C) OPERATING SYSTEM: MICROSOFT WORD FOR WINDOWS 95  
(D) SOFTWARE: MICROSOFT WORD VERSION 7.0A

## (v) CURRENT APPLICATION DATA:

(A) APPLICATION NUMBER:  
(B) FILING DATE:  
(C) CLASSIFICATION:

## (vi) PRIOR APPLICATION DATA:

(A) APPLICATION NUMBER: 60/030,601  
(B) FILING DATE: NOVEMBER 13, 1996

## (vii) ATTORNEY/AGENT INFORMATION:

(A) NAME: FLOYD, LINDA AXAMETHY  
(B) REGISTRATION NO.: 33,692  
(C) REFERENCE/DOCKET NUMBER: CR-9982

## (2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 1668 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:  
 (A) ORGANISM: DHAB1

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

ATGAAAAGAT CAAAACGATT TGCAGTACTG GCCCAGCGCC CCGTCAATCA GGACGGGCTG	60
ATTGGCGAGT GGCCTGAAGA GGGGCTGATC GCCATGGACA GGGGCTTTGA CCCGGTCTCT	120
TCAGTAAAAG TGGACAACGG TCTGATCGTC GAACTGGACG GCAAACGCCG GGACCAAGTTT	180
GACATGATCG ACCGATTTAT CGCCGATTAC GCGATCAACG TTGAGCGCAC AGAGCAGGCA	240
ATGCGCCTGG AGGCGGTGGA AATAGCCCGT ATGCTGGTGG ATATTACAGT CAGCCGGGAG	300
GAGATCATTG CCATCACTAC CGCCATCAGC CCGGCCAAAG CGGTGAGAGT GATGGCGCAG	360
ATGAACGTGG TGGAGATGAT GATGGCGCTG CAGAAGATGC GTGCCC GCCG GACCCCTCC	420
AACCAGTGCC ACGTCACCAA TCTCAAAGAT AATCCGGTGC AGATTGCCGC TGACGCCGCC	480
GAGGCCGGGA TCCGCGGCTT CTCAGAACAG GAGACCACGG TCGGTATCGC GCGCTACGCG	540
CCGTTTAACG CCCTGGCGCT GTTGGTCGGT TCGCAGTGCG GCCGCCCCGG CGTGTTGACG	600
CAGTGCTCGG TGAAGAGGC CACCGAGCTG GAGCTGGGCA TGGCTGGCTT AACCAGCTAC	660
GCCGAGACGG TGTGGTCTA CGGCACCGAA GCGGTATTTA CCGACGGCGA TGATACGCCG	720
TGGTCAAAGG CGTTCCTCGC CTCGGCCTAC GCCTCCCGCG GGTGAAAT GCGCTACACC	780
TCCGGCACCG GATCCGAAGC GCTGATGGGC TATTCGGAGA GCAAGTCGAT GCTCTACCTC	840
GAATCGCGCT GCATCTTCAT TACTAAAGGC GCCGGGGTTC AGGGACTGCA AAACGGCGCG	900
GTGAGCTGTA TCGGCATGAC CGGCGCTGTG CCGTCGGGCA TTCGGGCGGT GCTGGCGGAA	960
AACCTGATCG CCTCTATGCT CGACCTCGAA GTGGCGTCCG CCAACGACCA GACTTTCTCC	1020
CACTCGGATA TTCGCCGCAC CGCGCGCACC CTGATGCAGA TGCTGCCGGG CACCGACTTT	1080
ATTTTCTCCG GCTACAGCGC GGTGCCGAAC TACGACAACA TGTTCCGCCG CTCGAAGTTC	1140
GATGCGGAAG ATTTTGATGA TTACAACATC CTGCAGCGTG ACCTGATGGT TGACGGCGGC	1200
CTGCGTCCGG TGACCGAGGC GGAAACCAT GCCATTCCGC AGAAAGCGGC GCGGGCGATC	1260
CAGGCGGTTT TCCGCGAGCT GGGGCTGCCG CCAATCGCCG ACGAGGAGGT GGAGGCCGCC	1320
ACCTACGCGC ACGGCAGCAA CGAGATGCCG CCGCGTAACG TGGTGGAGGA TCTGAGTGCG	1380
GTGGAAGAGA TGATGAAGCG CAACATCACC GGCCTCGATA TTGTCGGCGC GCTGAGCCGC	1440

AGCGGCTTTG AGGATATCGC CAGCAATATT CTCAATATGC TGCGCCAGCG GGTCACCGGC 1500  
 GATTACCTGC AGACCTCGGC CATTCTCGAT CGGCAGTTGC AGGTGGTGAG TGCGGTCAAC 1560  
 GACATCAATG ACTATCAGGG GCCGGGCACC GGCTATCGCA TCTCTGCCGA ACGCTGGGCG 1620  
 GAGATCAAAA ATATTCCGGG CGTGGTTCAG CCCGACACCA TTGAATAA 1668

## (2) INFORMATION FOR SEQ ID NO:2:

- (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 585 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(vi) ORIGINAL SOURCE:  
 (A) ORGANISM: DHAB2

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

GTGCAACAGA CAACCCAAAT TCAGCCCTCT TTTACCCTGA AAACCCGCGA GGGCGGGGTA 60  
 GCTTCTGCCG ATGAACGCGC CGATGAAGTG GTGATCGGCG TCGGCCCTGC CTTTGATAAA 120  
 CACCAGCATC AACTCTGAT CGATATGCCC CATGGCGCGA TCCTCAAAGA GCTGATTGCC 180  
 GGGGTGGAAG AAGAGGGGCT TCACGCCCGG GTGGTGCGCA TTCTGCGCAC GTCCGACGTC 240  
 TCCTTTATGG CCTGGGATGC GGCCAACCTG AGCGGCTCGG GGATCGGCAT CGGTATCCAG 300  
 TCGAAGGGGA CCACGGTCAT CCATCAGCGC GATCTGCTGC CGCTCAGCAA CCTGGAGCTG 360  
 TTCTCCAGG CGCCGCTGCT GACGCTGGAG ACCTACCGGC AGATTGGCAA AAACGCTGCG 420  
 CGCTATGCGC GCAAAGAGTC ACCTTCGCCG GTGCCGGTGG TGAACGATCA GATGGTGCGG 480  
 CCGAAATTTA TGGCCAAAGC CGCGCTATTT CATATCAAAG AGACCAAACA TGTGGTGACG 540  
 GACGCCGAGC CCGTCACCCT GCACATCGAC TTAGTAAGGG AGTGA 585

## (2) INFORMATION FOR SEQ ID NO:3:

- (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 426 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(vi) ORIGINAL SOURCE:  
 (A) ORGANISM: DHAB3

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

ATGAGCGAGA AAACCATGCG CGTGCAGGAT TATCCGTTAG CCACCCGCTG CCCGGAGCAT 60  
 ATCCTGACGC CTACCGGCAA ACCATTGACC GATATTACCC TCGAGAAGGT GCTCTCTGGC 120  
 GAGGTGGGCC CGCAGGATGT GCGGATCTCC CGCCAGACCC TTGAGTACCA GGCGCAGATT 180  
 GCCGAGCAGA TGCAGCGCCA TGCGGTGGCG CGCAATTTCC GCCGCGCGGC GGAGCTTATC 240



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GCCATTCCTG ACGAGCGCAT TCTGGCTATC TATAACGCGC TCGCCCCGTT CCGCTCCTCG 300
CAGGCGGAGC TGCTGGCGAT CGCCGACGAG CTGGAGCACA CCTGGCATGC GACAGTGAAT 360
GCCGCCTTTG TCCGGGAGTC GCGGGAAGTG TATCAGCAGC GGCATAAGCT GCGTAAAGGA 420
AGCTAA 426

```

## (2) INFORMATION FOR SEQ ID NO:4:

- (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 1164 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(vi) ORIGINAL SOURCE:  
 (A) ORGANISM: DHAT

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

```

ATGAGCTATC GTATGTTTGA TTATCTGGTG CCAAACGTTA ACTTTTTTGG CCCCACGCC 60
ATTTCCGTAG TCGGCGAACG CTGCCAGCTG CTGGGGGGGA AAAAAGCCCT GCTGGTCACC 120
GACAAAGGCC TCGGGGCAAT TAAAGATGGC GCGGTGGACA AAACCCTGCA TTATCTGCGG 180
GAGGCCGGGA TCGAGGTGGC GATCTTTGAC GCGCTCGAGC CGAACCCGAA AGACACCAAC 240
GTGCGCGACG GCCTCGCCGT GTTTCGCCGC GAACAGTGCG ACATCATCGT CACCGTGGGC 300
GGCGGCAGCC CGCACGATTG CGGCAAAGGC ATCGGCATCG CCGCCACCCA TGAGGGCGAT 360
CTGTACCAGT ATGCCGGAAT CGAGACCCTG ACCAACCCGC TGCCGCCTAT CGTCGCGGTC 420
AATACCACCG CCGGCACCGC CAGCGAGGTC ACCCGCCACT GCGTCCTGAC CAACACCGAA 480
ACCAAAGTGA AGTTTGTGAT CGTCAGCTGG CGCAAACCTGC CGTCGGTCTC TATCAACGAT 540
CCACTGCTGA TGATCGGTAA ACCGGCCGCC CTGACCGCGG CGACCGGGAT GGATGCCCTG 600
ACCCACGCCG TAGAGGCCTA TATCTCCAAA GACGCTAACC CCGTGACGGA CGCCGCCGCC 660
ATGCAGGCGA TCCGCCTCAT CGCCCGCAAC CTGCGCCAGG CCGTGGCCCT CGGCAGCAAT 720
CTGCAGGCGC GGGAAAACAT GGCCTATGCT TCTCTGCTGG CCGGGATGGC TTTCAATAAC 780
GCCAACCTCG GCTACGTGCA CGCCATGGCG CACCAGCTGG GCGGCCTGTA CGACATGCCG 840
CACGGCGTGG CCAACGCTGT CCTGCTGCCG CATGTGGCGC GCTACAACCT GATCGCCAAC 900
CCGGAGAAAT TCGCCGATAT CGCTGAACTG ATGGGCGAAA ATATCACCGG ACTGTCCACT 960
CTCGACGCGG CGGAAAAGC CATCGCCGCT ATCACGCGTC TGTCGATGGA TATCGGTATT 1020
CCGCAGCATC TGCGCGATCT GGGGGTAAAA GAGGCCGACT TCCCCTACAT GGCGGAGATG 1080
GCTCTAAAAG ACGGCAATGC GTTCTCGAAC CCGCGTAAAG GCAACGAGCA GGAGATTGCC 1140
GCGATTTTCC GCCAGGCATT CTGA 1164

```

## (2) INFORMATION FOR SEQ ID NO:5:

- (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 1380 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(vi) ORIGINAL SOURCE:  
 (A) ORGANISM: GPD1

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

```

CTTTAATTTT CTTTATCTT ACTCTCCTAC ATAAGACATC AAGAAACAAT TGTATATTGT   60
ACACCCCCCC CCTCCACAAA CACAAATATT GATAATATAA AGATGTCTGC TGCTGCTGAT   120
AGATTAAACT TAACTTCCGG CCACTTGAAT GCTGGTAGAA AGAGAAGTTC CTCTTCTGTT   180
TCTTTGAAGG CTGCCGAAA GCCTTTCAAG GTTACTGTGA TTGGATCTGG TAACTGGGGT   240
ACTACTATTG CCAAGGTGGT TGCCGAAAAT TGTAAGGGAT ACCCAGAAGT TTTGCTCCA   300
ATAGTACAAA TGTGGGTGTT CGAAGAAGAG ATCAATGGTG AAAAATTGAC TGAAATCATA   360
AATACTAGAC ATCAAAACGT GAAATACTTG CCTGGCATCA CTCTACCCGA CAATTTGGTT   420
GCTAATCCAG ACTTGATTGA TTCAGTCAAG GATGTCGACA TCATCGTTTT CAACATTCCA   480
CATCAATTTT TGCCCCGTAT CTGTAGCCAA TTGAAAGGTC ATGTTGATTC ACACGTCAGA   540
GCTATCTCCT GTCTAAAGGG TTTTGAAGTT GGTGCTAAAG GTGTCCAATT GCTATCCTCT   600
TACATCACTG AGGAAC TAGG TATTCAATGT GGTGCTCTAT CTGGTGCTAA CATTGCCACC   660
GAAGTCGCTC AAGAACACTG GTCTGAAACA ACAGTTGCTT ACCACATTCC AAAGGATTTT   720
AGAGGCGAGG GCAAGGACGT CGACCATAAG GTTCTAAAGG CCTTGTTCCA CAGACCTTAC   780
TTCCACGTTA GTGTCATCGA AGATGTTGCT GGTATCTCCA TCTGTGGTGC TTTGAAGAAC   840
GTTGTTGCCT TAGGTTGTGG TTTCGTCGAA GGTCTAGGCT GGGGTAACAA CGCTTCTGCT   900
GCCATCCAAA GAGTCGGTTT GGGTGAGATC ATCAGATTCG GTCAAATGTT TTTCCAGAA   960
TCTAGAGAAG AAACATACTA CCAAGAGTCT GCTGGTGTTG CTGATTTGAT CACCACCTGC  1020
GCTGGTGGTA GAAACGTCAA GGTGCTAGG CTAATGGCTA CTTCTGGTAA GGACGCCTGG  1080
GAATGTGAAA AGGAGTTGTT GAATGGCCAA TCCGCTCAAG GTTTAATTAC CTGCAAAGAA  1140
GTTACGGAAT GGTGGAAC ATGTGGCTCT GTCGAAGACT TCCCATTATT TGAAGCCGTA  1200
TACCAAATCG TTACAACAA CTACCAATG AAGAACCTGC CGGACATGAT TGAAGAATTA  1260
GATCTACATG AAGATTAGAT TTATTGGAGA AAGATAACAT ATCATACTTC CCCCCTTTT  1320
TTGAGGCTC TTCTATATCA TATTCATAAA TTAGCATTAT GTCATTTCTC ATAACACTT  1380

```

## (2) INFORMATION FOR SEQ ID NO:6:

- (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 2946 base pairs  
 (B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(vi) ORIGINAL SOURCE:

(A) ORGANISM: GPD2

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

GAATTCGAGC	CTGAAGTGCT	GATTACCTTC	AGGTAGACTT	CATCTTGACC	CATCAACCCC	60
AGCGTCAATC	CTGCAAATAC	ACCACCCAGC	AGCACTAGGA	TGATAGAGAT	AATATAGTAC	120
GTGGTAACGC	TTGCCTCATC	ACCTACGCTA	TGGCCGGAAT	CGGCAACATC	CCTAGAATTG	180
AGTACGTGTG	ATCCGGATAA	CAACGGCAGT	GAATATATCT	TCGGTATCGT	AAAGATGTGA	240
TATAAGATGA	TGTATACCCA	ATGAGGAGCG	CCTGATCGTG	ACCTAGACCT	TAGTGGCAAA	300
AACGACATAT	CTATTATAGT	GGGGAGAGTT	TCGTGCAAAT	AACAGACGCA	GCAGCAAGTA	360
ACTGTGACGA	TATCAACTCT	TTTTTTATTA	TGTAATAAGC	AAACAAGCAC	GAATGGGGAA	420
AGCCTATGTG	CAATCACCAA	GGTCGTCCCT	TTTTTCCCCT	TTGCTAATTT	AGAATTTAAA	480
GAAACCAAAA	GAATGAAGAA	AGAAAACAAA	TACTAGCCCT	AACCCTGACT	TCGTTTCTAT	540
GATAATACCC	TGCTTTAATG	AACGGTATGC	CCTAGGGTAT	ATCTCACTCT	GTACGTTACA	600
AACTCCGTTT	ATTTTATCGG	AACATCCGAG	CACCCGCGCC	TTCTCAACC	CAGGCACCGC	660
CCCAGGTAAC	CGTGCGCGAT	GAGCTAATCC	TGAGCCATCA	CCCACCCAC	CCGTTGATGA	720
CAGCAATTGC	GGAGGGCGAA	AATAAACTG	GAGCAAGGAA	TTACCATCAC	CGTCACCATC	780
ACCATCATAT	CGCCTTAGCC	TCTAGCCATA	GCCATCATGC	AAGCGTGTAT	CTTCTAAGAT	840
TCAGTCATCA	TCATTACCGA	GTTTGTTTTT	CTTCACATGA	TGAAGAAGGT	TTGAGTATGC	900
TCGAAACAAT	AAGACGACGA	TGGCTCTGCC	ATTGGTTATA	TTACGCTTTT	GCGGCGAGGT	960
GCCGATGGGT	TGCTGAGGGG	AAGAGTGTTT	AGCTTACGGA	CCTATTGCCA	TTGTTATTCC	1020
GATTAATCTA	TTGTTACAGC	GCTCTTCTCT	ACCCTGTCAT	TCTAGTATTT	TTTTTTTTTT	1080
TTTTTGTTTT	TACTTTTTTT	TCTTCTTGCC	TTTTTTTCTT	GTTACTTTTT	TTCTAGTTTT	1140
TTTTCCTTCC	ACTAAGCTTT	TTCCTTGATT	TATCCTTGGG	TTCTTCTTTC	TACTCCTTTA	1200
GATTTTTTTT	TTATATATTA	ATTTTAAAGT	TTATGTATTT	TGGTAGATTC	AATTCTCTTT	1260
CCCTTTCCTT	TTCCTTCGCT	CCCCTTCTT	ATCAATGCTT	GCTGTCAGAA	GATTAACAAG	1320
ATACACATTC	CTTAAGCGAA	CGCATCCGGT	GTTATATACT	CGTCGTGCAT	ATAAAATTTT	1380
GCCTTCAAGA	TCTACTTTCC	TAAGAAGATC	ATTATTACAA	ACACAACGTC	ACTCAAAGAT	1440
GACTGCTCAT	ACTAATATCA	AACAGCACAA	ACACTGTCAT	GAGGACCATC	CTATCAGAAG	1500
ATCGGACTCT	GCCGTGTCAA	TTGTACATTT	GAAACGTGCG	CCCTTCAAGG	TTACAGTGAT	1560
TGGTTCTGGT	AACTGGGGGA	CCACCATCGC	CAAAGTCATT	GCGGAAAACA	CAGAATTGCA	1620
TTCCCATATC	TTCGAGCCAG	AGGTGAGAAT	GTGGGTTTTT	GATGAAAAGA	TCGGCGACGA	1680

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AAATCTGACG GATATCATAA ATACAAGACA CCAGAACGTT AAATATCTAC CCAATATTGA 1740
CCTGCCCCAT AATCTAGTGG CCGATCCTGA TCTTTTACAC TCCATCAAGG GTGCTGACAT 1800
CCTTGTTTTT AACATCCCTC ATCAATTTTT ACCAAACATA GTCAAACAAT TGCAAGGCCA 1860
CGTGGCCCTT CATGTAAGGG CCATCTCGTG TCTAAAAGGG TTCGAGTTGG GCTCCAAGGG 1920
TGTGCAATTG CTATCCTCCT ATGTTACTGA TGAGTTAGGA ATCCAATGTG GCGCACTATC 1980
TGGTGCAAAC TTGGCACC GG AAGTGGCCAA GGAGCATTGG TCCGAAACCA CCGTGGCTTA 2040
CCAAC TACCA AAGGATTATC AAGGTGATGG CAAGGATGTA GATCATAAGA TTTTGAAATT 2100
GCTGTTCCAC AGACCTTACT TCCACGTCAA TGTCATCGAT GATGTTGCTG GTATATCCAT 2160
TGCCGGTGCC TTGAAGAACG TCGTGGCACT TGCATGTGGT TTCGTAGAAG GSTATGGGATG 2220
GGGTAACAAT GCCTCCGCAG CCATTCAAAG GCTGGGTTTA GGTGAAATTA TCAAGTTCGG 2280
TAGAATGTTT TTCCAGAAT CCAAAGTCGA GACCTACTAT CAAGAATCCG CTGGTGTTGC 2340
AGATCTGATC ACCACCTGCT CAGGCGGTAG AAACGTCAAG GTTGCCACAT ACATGGCCAA 2400
GACCGGTAAG TCAGCCTTGG AAGCAGAAAA GGAATTGCTT AACGGTCAAT CCGCCCAAGG 2460
GATAATCACA TGCAGAGAAG TTCACGAGTG GCTACAAACA TGTGAGTTGA CCCAAGAATT 2520
CCCAATTATT CGAGGCAGTC TACCAGATAG TCTACAACAA CGTCCGCATG GAAGACCTAC 2580
CGGAGATGAT TGAAGAGCTA GACATCGATG ACGAATAGAC ACTCTCCCCC CCCCTCCCCC 2640
TCTGATCTTT CCTGTTGCCT CTTTTTCCCC CAACCAATTT ATCATTATAC ACAAGTTCTA 2700
CAACTACTAC TAGTAACATT ACTACAGTTA TTATAATTTT CTATTCTCTT TTTCTTTAAG 2760
AATCTATCAT TAACGTTAAT TTCTATATAT ACATAACTAC CATTATACAC GCTATTATCG 2820
TTTACATATC ACATCACCGT TAATGAAAGA TACGACACCC TGTACACTAA CACAATTAAA 2880
TAATCGCCAT AACCTTTTCT GTTATCTATA GCCCTTAAAG CTGTTTCTTC GAGCTTTTCA 2940
CTGCAG 2946

```

## (2) INFORMATION FOR SEQ ID NO:7:

- (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 3178 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(vi) ORIGINAL SOURCE:  
 (A) ORGANISM: GUT2

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

```

CTGCAGAACT TCGTCTGCTC TGTGCCCATC CTCGCGGTTA GAAAGAAGCT GAATTGTTTC 60
ATGCGCAAGG GCATCAGCGA GTGACCAATA ATCACTGCAC TAATTCCTTT TTAGCAACAC 120
ATACTTATAT ACAGCACCAG ACCTTATGTC TTTTCTCTGC TCCGATACGT TATCCCACCC 180
AACTTTTATT TCAGTTTTGG CAGGGGAAAT TTCACAACCC CGCACGCTAA AAATCGTATT 240

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TAAACTTAAA	AGAGAACAGC	CACAAATAGG	GAACTTTGGT	CTAAACGAAG	GACTCTCCCT	300
CCCTTATCTT	GACCGTGCTA	TTGCCATCAC	TGCTACAAGA	CTAAATACGT	ACTAATATAT	360
GTTTTCGGTA	ACGAGAAGAA	GAGCTGCCGG	TGCAGCTGCT	GCCATGGCCA	CAGCCACGGG	420
GACGCTGTAC	TGGATGACTA	GCCAAGGTGA	TAGGCCGTTA	GTGCACAATG	ACCCGAGCTA	480
CATGGTGCAA	TTCCCCACCG	CCGCTCCACC	GGCAGGTCTC	TAGACGAGAC	CTGCTGGACC	540
GTCTGGACAA	GACGCATCAA	TTGCACGTGT	TGATCATCGG	TGGCGGGGCC	ACGGGGACAG	600
GATGTGCCCT	AGATGCTGCG	ACCAGGGGAC	TCAATGTGGC	CCTTGTTGAA	AAGGGGGATT	660
TTGCCTCGGG	AACGTCGTCC	AAATCTACCA	AGATGATTCA	CGGTGGGGTG	CGGTACTTAG	720
AGAAGGCCCT	CTGGGAGTTC	TCCAAGGCAC	AAC TGGATCT	GGTCATCGAG	GCACTCAACG	780
AGCGTAAACA	TCTTATCAAC	ACTGCCCCCTC	ACCTGTGCAC	GGTGCTACCA	ATTCTGATCC	840
CCATCTACAG	CACCTGGCAG	GTCCCGTACA	TCTATATGGG	CTGTAAATTC	TACGATTTCT	900
TTGGCGGTTC	CCAAAAC TTG	AAAAAATCAT	ACCTACTGTC	CAAATCCGCC	ACCGTGGAGA	960
AGGCTCCCAT	GCTTACCACA	GACAATTTAA	AGGCCTCGCT	TGTGTACCAT	GATGGGTCCT	1020
TTAACGACTC	GCGTTTGAAC	GCCACTTTAG	CCATCACGGG	TGTGGAGAAC	GGCGCTACCG	1080
TCTTGATCTA	TGTCGAGSTA	CAAAAATTGA	TCAAAGACCC	AACTTCTGGT	AAGGTTATCG	1140
GTGCCGAGGC	CCGGGACGTT	GAGACTAATG	AGCTTGTGAG	AATCAACGCT	AAATGTGTGG	1200
TCAATGCCAC	GGGCCCATAC	AGTGACGCCA	TTTTGCAAAT	GGACCGCAAC	CCATCCGGTC	1260
TGCCGGACTC	CCCCTAAAC	GACAACTCCA	AGATCAAGTC	GACTTTCAAT	CAAATCTCCG	1320
TCATGGACCC	GAAAATGGTC	ATCCCATCTA	TTGGCGTTCA	CATCGTATTG	CCCTCTTTTT	1380
ACTCCCCGAA	GGATATGGGT	TTGTTGGACG	TCAGAACCTC	TGATGGCAGA	GTGATGTTCT	1440
TTTTACCTTG	GCAGGGCAAA	GTCCTTGCCG	GCACCACAGA	CATCCCCTA	AAGCAAGTCC	1500
CAGAAAACCC	TATGCCTACA	GAGGCTGATA	TTCAAGATAT	CTTGAAAGAA	CTACAGCACT	1560
ATATCGAATT	CCCCGTGAAA	AGAGAAGACG	TGCTAAGTGC	ATGGGCTGGT	GTCAGACCTT	1620
TGGTCAGAGA	TCCACGTACA	ATCCCCGCAG	ACGGGAAGAA	GGGCTCTGCC	ACTCAGGGCG	1680
TGGTAAGATC	CCACTTCTTG	TTCACTTCGG	ATAATGGCCT	AATTACTATT	GCAGGTGGTA	1740
AATGGACTAC	TTACAGACAA	ATGGCTGAGG	AAACAGTCGA	CAAAGTTGTC	GAAGTTGGCG	1800
GATTCCACAA	CCTGAAACCT	TGTCACACAA	GAGATATTAA	GCTTGCTGGT	GCAGAAGAAT	1860
GGACGCAAAA	CTATGTGGCT	TTATTGGCTC	AAA ACTACCA	TTTATCATCA	AAAATGTCCA	1920
ACTACTTGGT	TCAAAACTAC	GGAACCCGTT	CCTCTATCAT	TTGCGAATTT	TTCAAAGAAT	1980
CCATGGAAAA	TAAACTGCCT	TTGTCCTTAG	CCGACAAGGA	AAATAACGTA	ATCTACTCTA	2040
GCGAGGAGAA	CAACTTGGTC	AATTTTGATA	CTTTCAGATA	TCCATTCACA	ATCGGTGAGT	2100
TAAAGTATTC	CATGCAGTAC	GAATATTGTA	GAAC TCCCTT	GGACTTCCTT	TTAAGAAGAA	2160
CAAGATTCGC	CTTCTTGGAC	GCCAAGGAAG	CTTTGAATGC	CGTGCATGCC	ACCGTCAAAG	2220

TTATGGGTGA TGAGTTCAAT TGGTCGGAGA AAAAGAGGCA GTGGGAACTT GAAAAAACTG 2280  
 TGAAC TTCAT CCAAGGACGT TTCGGTGTCT AAATCGATCA TGATAGTTAA GGGTGACAAA 2340  
 GATAACATTC ACAAGAGTAA TAATAATGGT AATGATGATA ATAATAATAA TGATAGTAAT 2400  
 AACAAATAATA ATAATGGTGG TAATGGCAAT GAAATCGCTA TTATTACCTA TTTTCCTTAA 2460  
 TGGAAGAGTT AAAGTAACT AAAAAAACTA CAAAAATATA TGAAGAAAAA AAAAAAAGA 2520  
 GGTAAATAGAC TCTACTACTA CAATTGATCT TCAAATTATG ACCTTCCTAG TGTTTATATT 2580  
 CTATTTCCAA TACATAATAT AATCTATATA ATCATTGCTG GTAGACTTCC GTTTTAATAT 2640  
 CGTTTTAATT ATCCCCTTTA TCTCTAGTCT AGTTTTATCA TAAATATAG AACACTAAA 2700  
 TAATATTCTT CAAACGGTCC TGGTGCATAC GCAATACATA TTTATGGTGC AAAAAAAAAA 2760  
 ATGGAAAATT TTGCTAGTCA TAAACCCTTT CATAAAACAA TACGTAGACA TCGCTACTTG 2820  
 AAATTTTCAA GTTTTTATCA GATCCATGTT TCCTATCTGC CTTGACAACC TCATCGTCGA 2880  
 AATAGTACCA TTTAGAACGC CCAATATTCA CATTGTGTTC AAGGTCTTTA TTCACCAGTG 2940  
 ACGTGTAATG GCCATGATTA ATGTGCCTGT ATGGTTAACC ACTCCAAATA GCTTATATTT 3000  
 CATAGTGTCA TTGTTTTTCA ATATAATGTT TAGTATCAAT GGATATGTTA CGACGGTGTT 3060  
 ATTTTCTTG GTCAAATCGT AATAAAATCT CGATAAATGG ATGACTAAGA TTTTGGTAA 3120  
 AGTTACAAAA TTTATCGTTT TCACTGTTGT CAATTTTTTG TTCTTGTAAT CACTCGAG 3178

## (2) INFORMATION FOR SEQ ID NO:8:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 816 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: DNA (genomic)

## (vi) ORIGINAL SOURCE:

- (A) ORGANISM: GPP1

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

ATGAAACGTT TCAATGTTTT AAAATATATC AGAACAACAA AAGCAAATAT ACAAACCATC 60  
 GCAATGCCTT TGACCACAAA ACCTTTATCT TTGAAATCA ACGCCGCTCT ATTCGATGTT 120  
 GACGGTACCA TCATCATCTC TCAACCAGCC ATTGCTGCTT TCTGGAGAGA TTTCGGTAAA 180  
 GACAAGCCTT ACTTCGATGC CGAACACGTT ATTCACATCT CTCACGGTTG GAGAACTTAC 240  
 GATGCCATTG CCAAGTTCGC TCCAGACTTT GCTGATGAAG AATACGTAA CAAGCTAGAA 300  
 GGTGAAATCC CAGAAAAGTA CGGTGAACAC TCCATCGAAG TTCCAGGTGC TGTCAAGTTG 360  
 TGTAATGCTT TGAACGCCTT GCCAAAGGAA AAATGGGCTG TCGCCACCTC TGGTACCCGT 420  
 GACATGGCCA AGAAATGGTT CGACATTTTG AAGATCAAGA GACCAGAATA CTTATCACC 480  
 GCCAATGATG TCAAGCAAGG TAAGCCTCAC CCAGAACCAT ACTTAAAGGG TAGAAACGGT 540

TTGGGTTTCC CAATTAATGA ACAAGACCCA TCCAAATCTA AGGTTGTTGT CTTTGAAGAC 600  
 GCACCAGCTG GTATTGCTGC TGGTAAGGCT GCTGGCTGTA AAATCGTTGG TATTGCTACC 660  
 ACTTTCGATT TGGACTTCTT GAAGGAAAAG GGTGTGACA TCATTGTCAA GAACCACGAA 720  
 TCTATCAGAG TCGGTGAATA CAACGCTGAA ACCGATGAAG TCGAATTGAT CTTTGATGAC 780  
 TACTTATACG CTAAGGATGA CTTGTTGAAA TGGTAA 816

## (2) INFORMATION FOR SEQ ID NO:9:

- (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 753 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(vi) ORIGINAL SOURCE:  
 (A) ORGANISM: GPP2

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

ATGGGATTGA CTAATAAACC TCTATCTTTG AAAGTTAACG CCGCTTTGTT CGACGTCGAC 60  
 GGTACCATTA TCATCTCTCA ACCAGCCATT GCTGCATTCT GGAGGGATTT CGGTAAGGAC 120  
 AAACCTTATT TCGATGCTGA ACACGTTATC CAAGTCTCGC ATGGTTGGAG AACGTTTGAT 180  
 GCCATTGCTA AGTTCGCTCC AGACTTTGCC AATGAAGAGT ATGTTAACAA ATTAGAAGCT 240  
 GAAATTCGGG TCAAGTACGG TGAAAAATCC ATTGAAGTCC CAGGTGCAGT TAAGCTGTGC 300  
 AACGCTTTGA ACGCTCTACC AAAAGAGAAA TGGGCTGTGG CAACTTCGGG TACCCGTGAT 360  
 ATGGCACAAA AATGGTTCGA GCATCTGGGA ATCAGGAGAC CAAAGTACTT CATTACCGCT 420  
 AATGATGTCA AACAGGGTAA GCCTCATCCA GAACCATATC TGAAGGGCAG GAATGGCTTA 480  
 GGATATCCGA TCAATGAGCA AGACCCTTCC AAATCTAAGG TAGTAGTATT TGAAGACGCT 540  
 CCAGCAGGTA TTGCCGCCGG AAAAGCCGCC GGTGTGAAGA TCATTGGTAT TGCCACTACT 600  
 TTCGACTTGG ACTTCCTAAA GGAAAAAGGC TGTGACATCA TTGTCAAAAA CCACGAATCC 660  
 ATCAGAGTTG GCGGCTACAA TGCCGAAACA GACGAAGTTG AATTCATTTT TGACGACTAC 720  
 TTATATGCTA AGGACGATCT GTTGAAATGG TAA 753

## (2) INFORMATION FOR SEQ ID NO:10:

- (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 2520 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(vi) ORIGINAL SOURCE:  
 (A) ORGANISM: GUT1

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

TGTATTGGCC ACGATAACCA CCCTTTGTAT ACTGTTTTTG TTTTTCACAT GGTAATAAAC	60
GACTTTTATT AAACAACGTA TGTAAAAACA TAACAAGAAT CTACCCATAC AGGCCATTTT	120
GTAATTCTTC TCTCTAATT GGAGTAAAC CATCAATTAA AGGGTGTGGA GTAGCATAGT	180
GAGGGGCTGA CTGCATTGAC AAAAAAATG AAAAAAAAAA AGGAAAAGGA AAGGAAAAAA	240
AGACAGCCAA GACTTTTAGA ACGGATAAGG TGTAATAAAA TGTGGGGGGA TGCCTGTTCT	300
CGAACCATAT AAAATATACC ATGTGGTTTG AGTTGTGGCC GGAAGTATAC AAATAGTTAT	360
ATGTTTCCCT CTCTCTCCG ACTTGTAAGT TTCTCCAAAC GTTACATATT CCGATCAAGC	420
CAGCGCCTTT AACTAGTTT AAAACAAGAA CAGAGCCGTA TGTCCAAAAT AATGGAAGAT	480
TTACGAAGTG ACTACGTCCC GCTTATCGCC AGTATTGATG TAGGAACGAC CTCATCCAGA	540
TGCATTCTGT TCAACAGATG GGGCCAGGAC GTTTCAAAAC ACCAAATTGA ATATTCAACT	600
TCAGCATCGA AGGGCAAGAT TGGGGTGTCT GGCCTAAGGA GACCCTCTAC AGCCCCAGCT	660
CGTGAAACAC CAAACGCCGG TGACATCAA ACCAGCGGAA AGCCCATCTT TTCTGCAGAA	720
GGCTATGCCA TTCAAGAAAC CAAATTCCTA AAAATCGAGG AATTGGACTT GGACTTCCAT	780
AACGAACCCA CGTTGAAGTT CCCCAAACCG GGTGGGGTTG AGTGCCATCC GCAGAAATTA	840
CTGGTGAACG TCGTCCAATG CCTTGCCTCA AGTTTGCTCT CTCTGCAGAC TATCAACAGC	900
GAACGTGTAG CAAACGGTCT CCCACCTTAC AAGGTAATAT GCATGGGTAT AGCAAACATG	960
AGAGAAACCA CAATTCTGTG GTCCCGCCGC ACAGGAAAAC CAATTGTTAA CTACGGTATT	1020
GTTTGGAAACG ACACCAGAAC GATCAAATC GTTAGAGACA AATGGCAAAA CACTAGCGTC	1080
GATAGGCAAC TGCAGCTTAG ACAGAAGACT GGATTGCCAT TGCTCTCCAC GTATTTCTCC	1140
TGTTCCAAGC TGCCTGTGTT CCTCGACAAT GAGCCTCTGT GTACCAAGGC GTATGAGGAG	1200
AACGACCTGA TGTTCCGCAC TGTGGACACA TGGCTGATTT ACCAATTAAC TAAACAAAAG	1260
GCGTTCGTTT CTGACGTAAC CAACGCTTCC AGAACTGGAT TTATGAACCT CTCCACTTTA	1320
AAGTACGACA ACGAGTTGCT GGAATTTTGG GGTATTGACA AGAACCTGAT TCACATGCCC	1380
GAAATGTGT CCTCATCTCA ATACTACGGT GACTTTGGCA TTCCTGATTG GATAATGGAA	1440
AAGCTACACG ATTCGCCAAA AACAGTACTG CGAGATCTAG TCAAGAGAAA CCTGCCCATA	1500
CAGGGCTGTC TGGGCGACCA AAGCGCATCC ATGGTGGGGC AACTCGCTTA CAAACCCGGT	1560
GCTGCAAAAT GACTTTATGG TACCGGTTGC TTTTACTGT ACAATACGGG GACCAAAAAA	1620
TTGATCTCCC AACATGGCGC ACTGACGACT CTAGCATTTT GGTTCACACA TTTGCAAGAG	1680
TACGGTGGCC AAAAACCAGA ATTGAGCAAG CCACATTTG CATTAGAGGG TTCCGTCGCT	1740
GTGGCTGGTG CTGTGGTCCA ATGGCTACGT GATAATTTAC GATTGATCGA TAAATCAGAG	1800
GATGTCGGAC CGATTGCATC TACGGTTTCT GATTCTGGTG GCGTAGTTTT CGTCCCCGCA	1860
TTTAGTGGCC TATTCGCTCC CTATTGGGAC CCAGATGCCA GAGCCACCAT AATGGGGATG	1920



TCTCAATTCA CTA CTGCTC CCACATCGCC AGAGCTGCCG TGGAAGGTGT TTGCTTTCAA 1980  
 GCCAGGGCTA TCTTGAAGGC AATGAGTTCT GACGCGTTTG GTGAAGGTTC CAAAGACAGG 2040  
 GACTTTTTAG AGGAAATTTT CGACGTCACA TATGAAAAGT CGCCCTGTC GGTTCTGGCA 2100  
 GTGGATGGCG GGATGTCGAG GTCTAATGAA GTCATGCAAA TTCAAGCCGA TATCCTAGGT 2160  
 CCCTGTGTCA AAGTCAGAAG GTCTCCGACA GCGGAATGTA CCGCATTGGG GGCAGCCATT 2220  
 GCAGCCAATA TGGCTTTCAA GGATGTGAAC GAGCGCCCAT TATGGAAGGA CCTACACGAT 2280  
 GTTAAGAAAT GGGTCTTTTA CAATGGAATG GAGAAAAACG AACAAATATC ACCAGAGGCT 2340  
 CATCCAAACC TTAAGATATT CAGAAGTGAA TCCGACGATG CTGAAAGGAG AAAGCATTGG 2400  
 AAGTATTGGG AAGTTGCCGT GGAAAGATCC AAAGGTTGGC TGAAGGACAT AGAAGGTGAA 2460  
 CACGAACAGG TTCTAGAAAA CTTCCAATAA CAACATAAAT AATTCTATT AACAAATGTAA 2520

(2) INFORMATION FOR SEQ ID NO:11:

- (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 391 amino acids  
 (B) TYPE: amino acid  
 (C) STRANDEDNESS: unknown  
 (D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: protein

(vi) ORIGINAL SOURCE:  
 (A) ORGANISM: GPD1

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

Met	Ser	Ala	Ala	Ala	Asp	Arg	Leu	Asn	Leu	Thr	Ser	Gly	His	Leu	Asn	1	5	10	15
Ala	Gly	Arg	Lys	Arg	Ser	Ser	Ser	Ser	Val	Ser	Leu	Lys	Ala	Ala	Glu	20	25	30	
Lys	Pro	Phe	Lys	Val	Thr	Val	Ile	Gly	Ser	Gly	Asn	Trp	Gly	Thr	Thr	35	40	45	
Ile	Ala	Lys	Val	Val	Ala	Glu	Asn	Cys	Lys	Gly	Tyr	Pro	Glu	Val	Phe	50	55	60	
Ala	Pro	Ile	Val	Gln	Met	Trp	Val	Phe	Glu	Glu	Glu	Ile	Asn	Gly	Glu	65	70	75	80
Lys	Leu	Thr	Glu	Ile	Ile	Asn	Thr	Arg	His	Gln	Asn	Val	Lys	Tyr	Leu	85	90	95	
Pro	Gly	Ile	Thr	Leu	Pro	Asp	Asn	Leu	Val	Ala	Asn	Pro	Asp	Leu	Ile	100	105	110	
Asp	Ser	Val	Lys	Asp	Val	Asp	Ile	Ile	Val	Phe	Asn	Ile	Pro	His	Gln	115	120	125	
Phe	Leu	Pro	Arg	Ile	Cys	Ser	Gln	Leu	Lys	Gly	His	Val	Asp	Ser	His	130	135	140	
Val	Arg	Ala	Ile	Ser	Cys	Leu	Lys	Gly	Phe	Glu	Val	Gly	Ala	Lys	Gly	145	150	155	160

(2) INFORMATION FOR SEQ ID NO:12:

(A) LENGTH: 384 amino acids  
(B) TYPE: amino acid  
(C) STRANDEDNESS: unknown  
(D) TOPOLOGY: unknown

(vi) ORIGINAL SOURCE:  
(A) ORGANISM: GPD2

Met Thr Ala His Thr Asn Ile Lys Gln His Lys His Cys His Glu Asp  
1 5 10 15  
His Pro Ile Arg Arg Ser Asp Ser Ala Val Ser Ile Val His Leu Lys  
20 25 30

Arg Ala Pro Phe Lys Val Thr Val Ile Gly Ser Gly Asn Trp Gly Thr  
 35 40 45  
 Thr Ile Ala Lys Val Ile Ala Glu Asn Thr Glu Leu His Ser His Ile  
 50 55 60  
 Phe Glu Pro Glu Val Arg Met Trp Val Phe Asp Glu Lys Ile Gly Asp  
 65 70 75 80  
 Glu Asn Leu Thr Asp Ile Ile Asn Thr Arg His Gln Asn Val Lys Tyr  
 85 90 95  
 Leu Pro Asn Ile Asp Leu Pro His Asn Leu Val Ala Asp Pro Asp Leu  
 100 105 110  
 Leu His Ser Ile Lys Gly Ala Asp Ile Leu Val Phe Asn Ile Pro His  
 115 120 125  
 Gln Phe Leu Pro Asn Ile Val Lys Gln Leu Gln Gly His Val Ala Pro  
 130 135 140  
 His Val Arg Ala Ile Ser Cys Leu Lys Gly Phe Glu Leu Gly Ser Lys  
 145 150 155 160  
 Gly Val Gln Leu Leu Ser Ser Tyr Val Thr Asp Glu Leu Gly Ile Gln  
 165 170 175  
 Cys Gly Ala Leu Ser Gly Ala Asn Leu Ala Pro Glu Val Ala Lys Glu  
 180 185 190  
 His Trp Ser Glu Thr Thr Val Ala Tyr Gln Leu Pro Lys Asp Tyr Gln  
 195 200 205  
 Gly Asp Gly Lys Asp Val Asp His Lys Ile Leu Lys Leu Leu Phe His  
 210 215 220  
 Arg Pro Tyr Phe His Val Asn Val Ile Asp Asp Val Ala Gly Ile Ser  
 225 230 235 240  
 Ile Ala Gly Ala Leu Lys Asn Val Val Ala Leu Ala Cys Gly Phe Val  
 245 250 255  
 Glu Gly Met Gly Trp Gly Asn Asn Ala Ser Ala Ala Ile Gln Arg Leu  
 260 265 270  
 Gly Leu Gly Glu Ile Ile Lys Phe Gly Arg Met Phe Phe Pro Glu Ser  
 275 280 285  
 Lys Val Glu Thr Tyr Tyr Gln Glu Ser Ala Gly Val Ala Asp Leu Ile  
 290 295 300  
 Thr Thr Cys Ser Gly Gly Arg Asn Val Lys Val Ala Thr Tyr Met Ala  
 305 310 315 320  
 Lys Thr Gly Lys Ser Ala Leu Glu Ala Glu Lys Glu Leu Leu Asn Gly  
 325 330 335  
 Gln Ser Ala Gln Gly Ile Ile Thr Cys Arg Glu Val His Glu Trp Leu  
 340 345 350  
 Gln Thr Cys Glu Leu Thr Gln Glu Phe Pro Ile Ile Arg Gly Ser Leu  
 355 360 365  
 Pro Asp Ser Leu Gln Gln Arg Pro His Gly Arg Pro Thr Gly Asp Asp  
 370 375 380

## (2) INFORMATION FOR SEQ ID NO:13:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 614 amino acids  
 (B) TYPE: amino acid  
 (C) STRANDEDNESS: unknown  
 (D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: protein

## (vi) ORIGINAL SOURCE:

(A) ORGANISM: GUT2

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

```

Met Thr Arg Ala Thr Trp Cys Asn Ser Pro Pro Pro Leu His Arg Gln
1          5          10          15
Val Ser Arg Arg Asp Leu Leu Asp Arg Leu Asp Lys Thr His Gln Phe
20          25          30
Asp Val Leu Ile Ile Gly Gly Gly Ala Thr Gly Thr Gly Cys Ala Leu
35          40          45
Asp Ala Ala Thr Arg Gly Leu Asn Val Ala Leu Val Glu Lys Gly Asp
50          55          60
Phe Ala Ser Gly Thr Ser Ser Lys Ser Thr Lys Met Ile His Gly Gly
65          70          75          80
Val Arg Tyr Leu Glu Lys Ala Phe Trp Glu Phe Ser Lys Ala Gln Leu
85          90          95
Asp Leu Val Ile Glu Ala Leu Asn Glu Arg Lys His Leu Ile Asn Thr
100         105         110
Ala Pro His Leu Cys Thr Val Leu Pro Ile Leu Ile Pro Ile Tyr Ser
115         120         125
Thr Trp Gln Val Pro Tyr Ile Tyr Met Gly Cys Lys Phe Tyr Asp Phe
130         135         140
Phe Gly Gly Ser Gln Asn Leu Lys Lys Ser Tyr Leu Leu Ser Lys Ser
145         150         155         160
Ala Thr Val Glu Lys Ala Pro Met Leu Thr Thr Asp Asn Leu Lys Ala
165         170         175
Ser Leu Val Tyr His Asp Gly Ser Phe Asn Asp Ser Arg Leu Asn Ala
180         185         190
Thr Leu Ala Ile Thr Gly Val Glu Asn Gly Ala Thr Val Leu Ile Tyr
195         200         205
Val Glu Val Gln Lys Leu Ile Lys Asp Pro Thr Ser Gly Lys Val Ile
210         215         220
Gly Ala Glu Ala Arg Asp Val Glu Thr Asn Glu Leu Val Arg Ile Asn
225         230         235         240
Ala Lys Cys Val Val Asn Ala Thr Gly Pro Tyr Ser Asp Ala Ile Leu
245         250         255
Gln Met Asp Arg Asn Pro Ser Gly Leu Pro Asp Ser Pro Leu Asn Asp
260         265         270

```

Asn Ser Lys Ile Lys Ser Thr Phe Asn Gln Ile Ser Val Met Asp Pro  
 275 280 285  
 Lys Met Val Ile Pro Ser Ile Gly Val His Ile Val Leu Pro Ser Phe  
 290 295 300  
 Tyr Ser Pro Lys Asp Met Gly Leu Leu Asp Val Arg Thr Ser Asp Gly  
 305 310 315 320  
 Arg Val Met Phe Phe Leu Pro Trp Gln Gly Lys Val Leu Ala Gly Thr  
 325 330 335  
 Thr Asp Ile Pro Leu Lys Gln Val Pro Glu Asn Pro Met Pro Thr Glu  
 340 345 350  
 Ala Asp Ile Gln Asp Ile Leu Lys Glu Leu Gln His Tyr Ile Glu Phe  
 355 360 365  
 Pro Val Lys Arg Glu Asp Val Leu Ser Ala Trp Ala Gly Val Arg Pro  
 370 375 380  
 Leu Val Arg Asp Pro Arg Thr Ile Pro Ala Asp Gly Lys Lys Gly Ser  
 385 390 395 400  
 Ala Thr Gln Gly Val Val Arg Ser His Phe Leu Phe Thr Ser Asp Asn  
 405 410 415  
 Gly Leu Ile Thr Ile Ala Gly Gly Lys Trp Thr Thr Tyr Arg Gln Met  
 420 425 430  
 Ala Glu Glu Thr Val Asp Lys Val Val Glu Val Gly Gly Phe His Asn  
 435 440 445  
 Leu Lys Pro Cys His Thr Arg Asp Ile Lys Leu Ala Gly Ala Glu Glu  
 450 455 460  
 Trp Thr Gln Asn Tyr Val Ala Leu Leu Ala Gln Asn Tyr His Leu Ser  
 465 470 475 480  
 Ser Lys Met Ser Asn Tyr Leu Val Gln Asn Tyr Gly Thr Arg Ser Ser  
 485 490 495  
 Ile Ile Cys Glu Phe Phe Lys Glu Ser Met Glu Asn Lys Leu Pro Leu  
 500 505 510  
 Ser Leu Ala Asp Lys Glu Asn Asn Val Ile Tyr Ser Ser Glu Glu Asn  
 515 520 525  
 Asn Leu Val Asn Phe Asp Thr Phe Arg Tyr Pro Phe Thr Ile Gly Glu  
 530 535 540  
 Leu Lys Tyr Ser Met Gln Tyr Glu Tyr Cys Arg Thr Pro Leu Asp Phe  
 545 550 555 560  
 Leu Leu Arg Arg Thr Arg Phe Ala Phe Leu Asp Ala Lys Glu Ala Leu  
 565 570 575  
 Asn Ala Val His Ala Thr Val Lys Val Met Gly Asp Glu Phe Asn Trp  
 580 585 590  
 Ser Glu Lys Lys Arg Gln Trp Glu Leu Glu Lys Thr Val Asn Phe Ile  
 595 600 605  
 Gln Gly Arg Phe Gly Val  
 610

## (2) INFORMATION FOR SEQ ID NO:14:

- (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 339 amino acids  
 (B) TYPE: amino acid  
 (C) STRANDEDNESS: unknown  
 (D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: protein

- (vi) ORIGINAL SOURCE:  
 (A) ORGANISM: GPSA

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

```

Met Asn Gln Arg Asn Ala Ser Met Thr Val Ile Gly Ala Gly Ser Tyr
1          5          10          15
Gly Thr Ala Leu Ala Ile Thr Leu Ala Arg Asn Gly His Glu Val Val
20          25          30
Leu Trp Gly His Asp Pro Glu His Ile Ala Thr Leu Glu Arg Asp Arg
35          40          45
Cys Asn Ala Ala Phe Leu Pro Asp Val Pro Phe Pro Asp Thr Leu His
50          55          60
Leu Glu Ser Asp Leu Ala Thr Ala Leu Ala Ala Ser Arg Asn Ile Leu
65          70          75          80
Val Val Val Pro Ser His Val Phe Gly Glu Val Leu Arg Gln Ile Lys
85          90          95
Pro Leu Met Arg Pro Asp Ala Arg Leu Val Trp Ala Thr Lys Gly Leu
100         105         110
Glu Ala Glu Thr Gly Arg Leu Leu Gln Asp Val Ala Arg Glu Ala Leu
115         120         125
Gly Asp Gln Ile Pro Leu Ala Val Ile Ser Gly Pro Thr Phe Ala Lys
130         135         140
Glu Leu Ala Ala Gly Leu Pro Thr Ala Ile Ser Leu Ala Ser Thr Asp
145         150         155         160
Gln Thr Phe Ala Asp Asp Leu Gln Gln Leu Leu His Cys Gly Lys Ser
165         170         175
Phe Arg Val Tyr Ser Asn Pro Asp Phe Ile Gly Val Gln Leu Gly Gly
180         185         190
Ala Val Lys Asn Val Ile Ala Ile Gly Ala Gly Met Ser Asp Gly Ile
195         200         205
Gly Phe Gly Ala Asn Ala Arg Thr Ala Leu Ile Thr Arg Gly Leu Ala
210         215         220
Glu Met Ser Arg Leu Gly Ala Ala Leu Gly Ala Asp Pro Ala Thr Phe
225         230         235         240
Met Gly Met Ala Gly Leu Gly Asp Leu Val Leu Thr Cys Thr Asp Asn
245         250         255
Gln Ser Arg Asn Arg Arg Phe Gly Met Met Leu Gly Gln Gly Met Asp
260         265         270

```

Val Gln Ser Ala Gln Glu Lys Ile Gly Gln Val Val Glu Gly Tyr Arg  
 275 280 285

Asn Thr Lys Glu Val Arg Glu Leu Ala His Arg Phe Gly Val Glu Met  
 290 295 300

Pro Ile Thr Glu Glu Ile Tyr Gln Val Leu Tyr Cys Gly Lys Asn Ala  
 305 310 315 320

Arg Glu Ala Ala Leu Thr Leu Leu Gly Arg Ala Arg Lys Asp Glu Arg  
 325 330 335

Ser Ser His

## (2) INFORMATION FOR SEQ ID NO:15:

- (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 501 amino acids  
 (B) TYPE: amino acid  
 (C) STRANDEDNESS: unknown  
 (D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: protein

(vi) ORIGINAL SOURCE:  
 (A) ORGANISM: GLPD

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

Met Glu Thr Lys Asp Leu Ile Val Ile Gly Gly Gly Ile Asn Gly Ala  
 1 5 10 15

Gly Ile Ala Ala Asp Ala Ala Gly Arg Gly Leu Ser Val Leu Met Leu  
 20 25 30

Glu Ala Gln Asp Leu Ala Cys Ala Thr Ser Ser Ala Ser Ser Lys Leu  
 35 40 45

Ile His Gly Gly Leu Arg Tyr Leu Glu His Tyr Glu Phe Arg Leu Val  
 50 55 60

Ser Glu Ala Leu Ala Glu Arg Glu Val Leu Leu Lys Met Ala Pro His  
 65 70 75 80

Ile Ala Phe Pro Met Arg Phe Arg Leu Pro His Arg Pro His Leu Arg  
 85 90 95

Pro Ala Trp Met Ile Arg Ile Gly Leu Phe Met Tyr Asp His Leu Gly  
 100 105 110

Lys Arg Thr Ser Leu Pro Gly Ser Thr Gly Leu Arg Phe Gly Ala Asn  
 115 120 125

Ser Val Leu Lys Pro Glu Ile Lys Arg Gly Phe Glu Tyr Ser Asp Cys  
 130 135 140

Trp Val Asp Asp Ala Arg Leu Val Leu Ala Asn Ala Gln Met Val Val  
 145 150 155 160

Arg Lys Gly Gly Glu Val Leu Thr Arg Thr Arg Ala Thr Ser Ala Arg  
 165 170 175

Arg Glu Asn Gly Leu Trp Ile Val Glu Ala Glu Asp Ile Asp Thr Gly  
 180 185 190

Lys Lys Tyr Ser Trp Gln Ala Arg Gly Leu Val Asn Ala Thr Gly Pro  
 195 200 205  
 Trp Val Lys Gln Phe Phe Asp Asp Gly Met His Leu Pro Ser Pro Tyr  
 210 215 220  
 Gly Ile Arg Leu Ile Lys Gly Ser His Ile Val Val Pro Arg Val His  
 225 230 235 240  
 Thr Gln Lys Gln Ala Tyr Ile Leu Gln Asn Glu Asp Lys Arg Ile Val  
 245 250 255  
 Phe Val Ile Pro Trp Met Asp Glu Phe Ser Ile Ile Gly Thr Thr Asp  
 260 265 270  
 Val Glu Tyr Lys Gly Asp Pro Lys Ala Val Lys Ile Glu Glu Ser Glu  
 275 280 285  
 Ile Asn Tyr Leu Leu Asn Val Tyr Asn Thr His Phe Lys Lys Gln Leu  
 290 295 300  
 Ser Arg Asp Asp Ile Val Trp Thr Tyr Ser Gly Val Arg Pro Leu Cys  
 305 310 315 320  
 Asp Asp Glu Ser Asp Ser Pro Gln Ala Ile Thr Arg Asp Tyr Thr Leu  
 325 330 335  
 Asp Ile His Asp Glu Asn Gly Lys Ala Pro Leu Leu Ser Val Phe Gly  
 340 345 350  
 Gly Lys Leu Thr Thr Tyr Arg Lys Leu Ala Glu His Ala Leu Glu Lys  
 355 360 365  
 Leu Thr Pro Tyr Tyr Gln Gly Ile Gly Pro Ala Trp Thr Lys Glu Ser  
 370 375 380  
 Val Leu Pro Gly Gly Ala Ile Glu Gly Asp Arg Asp Asp Tyr Ala Ala  
 385 390 395 400  
 Arg Leu Arg Arg Arg Tyr Pro Phe Leu Thr Glu Ser Leu Ala Arg His  
 405 410 415  
 Tyr Ala Arg Thr Tyr Gly Ser Asn Ser Glu Leu Leu Leu Gly Asn Ala  
 420 425 430  
 Gly Thr Val Ser Asp Leu Gly Glu Asp Phe Gly His Glu Phe Tyr Glu  
 435 440 445  
 Ala Glu Leu Lys Tyr Leu Val Asp His Glu Trp Val Arg Arg Ala Asp  
 450 455 460  
 Asp Ala Leu Trp Arg Arg Thr Lys Gln Gly Met Trp Leu Asn Ala Asp  
 465 470 475 480  
 Gln Gln Ser Arg Val Ser Gln Trp Leu Val Glu Tyr Thr Gln Gln Arg  
 485 490 495  
 Leu Ser Leu Ala Ser  
 500

(2) INFORMATION FOR SEQ ID NO:16:

- (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 542 amino acids  
 (B) TYPE: amino acid



(C) STRANDEDNESS: unknown  
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: protein

(vi) ORIGINAL SOURCE:  
(A) ORGANISM: GLPABC

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

```

Met Lys Thr Arg Asp Ser Gln Ser Ser Asp Val Ile Ile Ile Gly Gly
1      5      10      15
Gly Ala Thr Gly Ala Gly Ile Ala Arg Asp Cys Ala Leu Arg Gly Leu
20      25      30
Arg Val Ile Leu Val Glu Arg His Asp Ile Ala Thr Gly Ala Thr Gly
35      40      45
Arg Asn His Gly Leu Leu His Ser Gly Ala Arg Tyr Ala Val Thr Asp
50      55      60
Ala Glu Ser Ala Arg Glu Cys Ile Ser Glu Asn Gln Ile Leu Lys Arg
65      70      75      80
Ile Ala Arg His Cys Val Glu Pro Thr Asn Gly Leu Phe Ile Thr Leu
85      90      95
Pro Glu Asp Asp Leu Ser Phe Gln Ala Thr Phe Ile Arg Ala Cys Glu
100     105     110
Glu Ala Gly Ile Ser Ala Glu Ala Ile Asp Pro Gln Gln Ala Arg Ile
115     120     125
Ile Glu Pro Ala Val Asn Pro Ala Leu Ile Gly Ala Val Lys Val Pro
130     135     140
Asp Gly Thr Val Asp Pro Phe Arg Leu Thr Ala Ala Asn Met Leu Asp
145     150     155     160
Ala Lys Glu His Gly Ala Val Ile Leu Thr Ala His Glu Val Thr Gly
165     170     175
Leu Ile Arg Glu Gly Ala Thr Val Cys Gly Val Arg Val Arg Asn His
180     185     190
Leu Thr Gly Glu Thr Gln Ala Leu His Ala Pro Val Val Val Asn Ala
195     200     205
Ala Gly Ile Trp Gly Gln His Ile Ala Glu Tyr Ala Asp Leu Arg Ile
210     215     220
Arg Met Phe Pro Ala Lys Gly Ser Leu Leu Ile Met Asp His Arg Ile
225     230     235     240
Asn Gln His Val Ile Asn Arg Cys Arg Lys Pro Ser Asp Ala Asp Ile
245     250     255
Leu Val Pro Gly Asp Thr Ile Ser Leu Ile Gly Thr Thr Ser Leu Arg
260     265     270
Ile Asp Tyr Asn Glu Ile Asp Asp Asn Arg Val Thr Ala Glu Glu Val
275     280     285
Asp Ile Leu Leu Arg Glu Gly Glu Lys Leu Ala Pro Val Met Ala Lys
290     295     300

```

Thr Arg Ile Leu Arg Ala Tyr Ser Gly Val Arg Pro Leu Val Ala Ser  
 305 310 315 320  
 Asp Asp Asp Pro Ser Gly Arg Asn Leu Ser Arg Gly Ile Val Leu Leu  
 325 330 335  
 Asp His Ala Glu Arg Asp Gly Leu Asp Gly Phe Ile Thr Ile Thr Gly  
 340 345 350  
 Gly Lys Leu Met Thr Tyr Arg Leu Met Ala Glu Trp Ala Thr Asp Ala  
 355 360 365  
 Val Cys Arg Lys Leu Gly Asn Thr Arg Pro Cys Thr Thr Ala Asp Leu  
 370 375 380  
 Ala Leu Pro Gly Ser Gln Glu Pro Ala Glu Val Thr Leu Arg Lys Val  
 385 390 395 400  
 Ile Ser Leu Pro Ala Pro Leu Arg Gly Ser Ala Val Tyr Arg His Gly  
 405 410 415  
 Asp Arg Thr Pro Ala Trp Leu Ser Glu Gly Arg Leu His Arg Ser Leu  
 420 425 430  
 Val Cys Glu Cys Glu Ala Val Thr Ala Gly Glu Val Gln Tyr Ala Val  
 435 440 445  
 Glu Asn Leu Asn Val Asn Ser Leu Leu Asp Leu Arg Arg Arg Thr Arg  
 450 455 460  
 Val Gly Met Gly Thr Cys Gln Gly Glu Leu Cys Ala Cys Arg Ala Ala  
 465 470 475 480  
 Gly Leu Leu Gln Arg Phe Asn Val Thr Thr Ser Ala Gln Ser Ile Glu  
 485 490 495  
 Gln Leu Ser Thr Phe Leu Asn Glu Arg Trp Lys Gly Val Gln Pro Ile  
 500 505 510  
 Ala Trp Gly Asp Ala Leu Arg Glu Ser Glu Phe Thr Arg Trp Val Tyr  
 515 520 525  
 Gln Gly Leu Cys Gly Leu Glu Lys Glu Gln Lys Asp Ala Leu  
 530 535 540

## (2) INFORMATION FOR SEQ ID NO:17:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 250 amino acids
  - (B) TYPE: amino acid
  - (C) STRANDEDNESS: unknown
  - (D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: protein

(vi) ORIGINAL SOURCE:

- (A) ORGANISM: GPP2

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

Met Gly Leu Thr Thr Lys Pro Leu Ser Leu Lys Val Asn Ala Ala Leu  
 1 5 10 15  
 Phe Asp Val Asp Gly Thr Ile Ile Ile Ser Gln Pro Ala Ile Ala Ala  
 20 25 30

Phe Trp Arg Asp Phe Gly Lys Asp Lys Pro Tyr Phe Asp Ala Glu His  
           35                          40                          45  
 Val Ile Gln Val Ser His Gly Trp Arg Thr Phe Asp Ala Ile Ala Lys  
           50                          55                          60  
 Phe Ala Pro Asp Phe Ala Asn Glu Glu Tyr Val Asn Lys Leu Glu Ala  
   65                          70                          75                          80  
 Glu Ile Pro Val Lys Tyr Gly Glu Lys Ser Ile Glu Val Pro Gly Ala  
                           85                          90                          95  
 Val Lys Leu Cys Asn Ala Leu Asn Ala Leu Pro Lys Glu Lys Trp Ala  
                          100                         105                         110  
 Val Ala Thr Ser Gly Thr Arg Asp Met Ala Gln Lys Trp Phe Glu His  
          115                         120                         125  
 Leu Gly Ile Arg Arg Pro Lys Tyr Phe Ile Thr Ala Asn Asp Val Lys  
  130                         135                         140  
 Gln Gly Lys Pro His Pro Glu Pro Tyr Leu Lys Gly Arg Asn Gly Leu  
  145                         150                         155                         160  
 Gly Tyr Pro Ile Asn Glu Gln Asp Pro Ser Lys Ser Lys Val Val Val  
                          165                         170                         175  
 Phe Glu Asp Ala Pro Ala Gly Ile Ala Ala Gly Lys Ala Ala Gly Cys  
          180                         185                         190  
 Lys Ile Ile Gly Ile Ala Thr Thr Phe Asp Leu Asp Phe Leu Lys Glu  
          195                         200                         205  
 Lys Gly Cys Asp Ile Ile Val Lys Asn His Glu Ser Ile Arg Val Gly  
  210                         215                         220  
 Gly Tyr Asn Ala Glu Thr Asp Glu Val Glu Phe Ile Phe Asp Asp Tyr  
  225                         230                         235                         240  
 Leu Tyr Ala Lys Asp Asp Leu Leu Lys Trp  
                          245                         250

## (2) INFORMATION FOR SEQ ID NO:18:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 709 amino acids
  - (B) TYPE: amino acid
  - (C) STRANDEDNESS: unknown
  - (D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: protein

(vi) ORIGINAL SOURCE:

- (A) ORGANISM: GUT1

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

Met Phe Pro Ser Leu Phe Arg Leu Val Val Phe Ser Lys Arg Tyr Ile  
 1                          5                          10                          15  
 Phe Arg Ser Ser Gln Arg Leu Tyr Thr Ser Leu Lys Gln Glu Gln Ser  
          20                         25                         30  
 Arg Met Ser Lys Ile Met Glu Asp Leu Arg Ser Asp Tyr Val Pro Leu  
  35                         40                         45

Ile Ala Ser Ile Asp Val Gly Thr Thr Ser Ser Arg Cys Ile Leu Phe  
 50 55 60  
 Asn Arg Trp Gly Gln Asp Val Ser Lys His Gln Ile Glu Tyr Ser Thr  
 65 70 75 80  
 Ser Ala Ser Lys Gly Lys Ile Gly Val Ser Gly Leu Arg Arg Pro Ser  
 85 90 95  
 Thr Ala Pro Ala Arg Glu Thr Pro Asn Ala Gly Asp Ile Lys Thr Ser  
 100 105 110  
 Gly Lys Pro Ile Phe Ser Ala Glu Gly Tyr Ala Ile Gln Glu Thr Lys  
 115 120 125  
 Phe Leu Lys Ile Glu Glu Leu Asp Leu Asp Phe His Asn Glu Pro Thr  
 130 135 140  
 Leu Lys Phe Pro Lys Pro Gly Trp Val Glu Cys His Pro Gln Lys Leu  
 145 150 155 160  
 Leu Val Asn Val Val Gln Cys Leu Ala Ser Ser Leu Leu Ser Leu Gln  
 165 170 175  
 Thr Ile Asn Ser Glu Arg Val Ala Asn Gly Leu Pro Pro Tyr Lys Val  
 180 185 190  
 Ile Cys Met Gly Ile Ala Asn Met Arg Glu Thr Thr Ile Leu Trp Ser  
 195 200 205  
 Arg Arg Thr Gly Lys Pro Ile Val Asn Tyr Gly Ile Val Trp Asn Asp  
 210 215 220  
 Thr Arg Thr Ile Lys Ile Val Arg Asp Lys Trp Gln Asn Thr Ser Val  
 225 230 235 240  
 Asp Arg Gln Leu Gln Leu Arg Gln Lys Thr Gly Leu Pro Leu Leu Ser  
 245 250 255  
 Thr Tyr Phe Ser Cys Ser Lys Leu Arg Trp Phe Leu Asp Asn Glu Pro  
 260 265 270  
 Leu Cys Thr Lys Ala Tyr Glu Glu Asn Asp Leu Met Phe Gly Thr Val  
 275 280 285  
 Asp Thr Trp Leu Ile Tyr Gln Leu Thr Lys Gln Lys Ala Phe Val Ser  
 290 295 300  
 Asp Val Thr Asn Ala Ser Arg Thr Gly Phe Met Asn Leu Ser Thr Leu  
 305 310 315 320  
 Lys Tyr Asp Asn Glu Leu Leu Glu Phe Trp Gly Ile Asp Lys Asn Leu  
 325 330 335  
 Ile His Met Pro Glu Ile Val Ser Ser Ser Gln Tyr Tyr Gly Asp Phe  
 340 345 350  
 Gly Ile Pro Asp Trp Ile Met Glu Lys Leu His Asp Ser Pro Lys Thr  
 355 360 365  
 Val Leu Arg Asp Leu Val Lys Arg Asn Leu Pro Ile Gln Gly Cys Leu  
 370 375 380  
 Gly Asp Gln Ser Ala Ser Met Val Gly Gln Leu Ala Tyr Lys Pro Gly  
 385 390 395 400

Ala Ala Lys Cys Thr Tyr Gly Thr Gly Cys Phe Leu Leu Tyr Asn Thr  
 405 410 415  
 Gly Thr Lys Lys Leu Ile Ser Gln His Gly Ala Leu Thr Thr Leu Ala  
 420 425 430  
 Phe Trp Phe Pro His Leu Gln Glu Tyr Gly Gly Gln Lys Pro Glu Leu  
 435 440 445  
 Ser Lys Pro His Phe Ala Leu Glu Gly Ser Val Ala Val Ala Gly Ala  
 450 455 460  
 Val Val Gln Trp Leu Arg Asp Asn Leu Arg Leu Ile Asp Lys Ser Glu  
 465 470 475 480  
 Asp Val Gly Pro Ile Ala Ser Thr Val Pro Asp Ser Gly Gly Val Val  
 485 490 495  
 Phe Val Pro Ala Phe Ser Gly Leu Phe Ala Pro Tyr Trp Asp Pro Asp  
 500 505 510  
 Ala Arg Ala Thr Ile Met Gly Met Ser Gln Phe Thr Thr Ala Ser His  
 515 520 525  
 Ile Ala Arg Ala Ala Val Glu Gly Val Cys Phe Gln Ala Arg Ala Ile  
 530 535 540  
 Leu Lys Ala Met Ser Ser Asp Ala Phe Gly Glu Gly Ser Lys Asp Arg  
 545 550 555 560  
 Asp Phe Leu Glu Glu Ile Ser Asp Val Thr Tyr Glu Lys Ser Pro Leu  
 565 570 575  
 Ser Val Leu Ala Val Asp Gly Gly Met Ser Arg Ser Asn Glu Val Met  
 580 585 590  
 Gln Ile Gln Ala Asp Ile Leu Gly Pro Cys Val Lys Val Arg Arg Ser  
 595 600 605  
 Pro Thr Ala Glu Cys Thr Ala Leu Gly Ala Ala Ile Ala Ala Asn Met  
 610 615 620  
 Ala Phe Lys Asp Val Asn Glu Arg Pro Leu Trp Lys Asp Leu His Asp  
 625 630 635 640  
 Val Lys Lys Trp Val Phe Tyr Asn Gly Met Glu Lys Asn Glu Gln Ile  
 645 650 655  
 Ser Pro Glu Ala His Pro Asn Leu Lys Ile Phe Arg Ser Glu Ser Asp  
 660 665 670  
 Asp Ala Glu Arg Arg Lys His Trp Lys Tyr Trp Glu Val Ala Val Glu  
 675 680 685  
 Arg Ser Lys Gly Trp Leu Lys Asp Ile Glu Gly Glu His Glu Gln Val  
 690 695 700  
 Leu Glu Asn Phe Gln  
 705

(2) INFORMATION FOR SEQ ID NO:19:

- (1) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 12145 base pairs  
 (B) TYPE: nucleic acid

- (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: DNA (genomic)
- (vi) ORIGINAL SOURCE:  
 (A) ORGANISM: PHK28-26
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

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GTCGACCACC ACGGTGGTGA CTTTAATGCC GCTCTCATGC AGCAGCTCGG TGGCGGTCTC   60
AAAATTCAGG ATGTCGCCGG TATAGTTTTT GATAATCAGC AAGACGCCTT CGCCGCCGTC   120
AATTTGCATC GCGCATTCAA ACATTTTGTG CGGCGTCGCG GAGGTGAATA TTTCCCCCGG   180
ACAGGCGCCG GAGAGCATGC CCTGGCCGAT ATAGCCGCAG TGCATCGGTT CATGTCCGCT   240
GCCGCCGCCG GAGAGCAGGG CCACCTTGCC AGCCACCGGC GCGTCGGTGC GGGTCACATA   300
CAGCGGGTCC TGATGCAGGG TCAGCTGCGG ATGGGCTTTA GCCAGCCCCT GTAATTGTTC   360
ATTCAGTACA TCTTCAACAC GGTTAATCAG CTTTTTCATT ATTCAGTGCT CCGTTGGAGA   420
AGGTTTCGATG CCGCCTCTCT GCTGGCGGAG GCGGTCATCG CGTAGGGGTA TCGTCTGACG   480
GTGGAGCGTG CCTGGCGATA TGATGATTCT GGCTGAGCGG ACGAAAAAAA GAATGCCCCG   540
ACGATCGGGT TTCATTACGA AACATTGCTT CCTGATTTTG TTTCTTTATG GAACGTTTTT   600
GCTGAGGATA TGGTGAAAAT GCGAGCTGGC GCGCTTTTTT TCTTCTGCCA TAAGCGGCGG   660
TCAGGATAGC CGGCGAAGCG GGTGGGAAAA AATTTTTTGC TGATTTTCTG CCGACTGCGG   720
GAGAAAAGGC GGTCAAACAC GGAGGATTGT AAGGGCATTG TGCGGCAAAG GAGCGGATCG   780
GGATCGCAAT CCTGACAGAG ACTAGGGTTT TTTGTTCCAA TATGGAACGT AAAAAATTAA   840
CCTGTGTTTC ATATCAGAAC AAAAAGGCGA AAGATTTTTT TGTTCCCTGC CGGCCCTACA   900
GTGATCGCAC TGCTCCGGTA CGCTCCGTTC AGGCCGCGCT TCACTGGCCG GCGCGGATAA   960
CGCCAGGGCT CATCATGTCT ACATGCGCAC TTATTTGAGG GTGAAAGGAA TGCTAAAGT   1020
TATTCAATCT CCAGCCAAAT ATCTTCAGGG TCCTGATGCT GCTGTTCTGT TCGGTCAATA   1080
TGCCAAAAAC CTGGCGGAGA GCTTCTTCGT CATCGCTGAC GATTTCTGTA TGAAGCTGGC   1140
GGGAGAGAAA GTGGTGAATG GCCTGCAGAG CCACGATATT CGCTGCCATG CGGAACGGTT   1200
TAACGGCGAA TGCAGCCATG CGGAAATCAA CCGTCTGATG GCGATTTTGC AAAAACAGGG   1260
CTGCCGCGGC GTGGTCGGGA TCGGCGGTGG TAAACCCTC GATACCGCGA AGGCGATCGG   1320
TTACTACCAG AAGCTGCCGG TGGTGGTGAT CCCGACCATC GCCTCGACCG ATGCGCCAAC   1380
CAGCGCGCTG TCGGTGATCT ACACCGAAGC GGGCGAGTTT GAAGAGTATC TGATCTATCC   1440
GAAAAACCCG GATATGGTGG TGATGGACAC GCGGATTATC GCCAAAGCGC CGGTACGCCT   1500
GCTGGTCTCC GGCATGGGCG ATGCGCTCTC CACCTGGTTC GAGGCCAAAG CTTGCTACGA   1560
TGCGCGCGCC ACCAGCATGG CCGGAGGACA GTCCACCGAG GCGGCGCTGA GCCTCGCCCG   1620
CCTGTGCTAT GATACGCTGC TGGCGGAGGG CGAAAAGGCC CGTCTGGCGG CGCAGGCCGG   1680

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GGTAGTGACC	GAAGCGCTGG	AGCGCATCAT	CGAGGCGAAC	ACTTACCTCA	GCGGCATTGG	1740
CTTTGAAAGC	AGTGGCCTGG	CCGCTGCCCC	TGCAATCCAC	AACGGTTTCA	CCATTCTTGA	1800
AGAGTGCCAT	CACCTGTATC	ACGGTGAGAA	AGTGGCCTTC	GGTACCCTGG	CGCAGCTGGT	1860
GCTGCAGAAC	AGCCCGATGG	ACGAGATTGA	AACGGTGCAG	GGCTTCTGCC	AGCGCGTCGG	1920
CCTGCCGGTG	ACGCTCGCGC	AGATGGGCGT	CAAAGAGGGG	ATCGACGAGA	AAATCGCCGC	1980
GGTGGCGAAA	GCTACCTGCG	CGGAAGGGGA	AACCATCCAT	AATATGCCGT	TTGCGGTGAC	2040
CCCGGAGAGC	GTCCATGCCG	CTATCCTCAC	CGCCGATCTG	TTAGGCCAGC	AGTGGCTGGC	2100
GCGTTAATTC	GCGGTGGCTA	AACCGCTGGC	CCAGGTCAGC	GGTTTTTCTT	TCTCCCCTCC	2160
GGCAGTCGCT	GCCGGAGGGG	TTCTCTATGG	TACAACGCGG	AAAAGGATAT	GACTGTTCAG	2220
ACTCAGGATA	CCGGAAGGC	GGTCTCTTCC	GTCATTGCCC	AGTCATGGCA	CCGCTGCAGC	2280
AAGTTTATGC	AGCGCGAAAC	CTGGCAAACG	CCGCACCAGG	CCCAGGGCCT	GACCTTCGAC	2340
TCCATCTGTC	GGCGTAAAC	CGCGCTGCTC	ACCATCGGCC	AGGCGGCGCT	GGAAGACGCC	2400
TGGGAGTTTA	TGGACGGCCG	CCCCTGCGCG	CTGTTTATTC	TTGATGAGTC	CGCCTGCATC	2460
CTGAGCCGTT	GCGGCGAGCC	GCAAACCCTG	GCCCAGCTGG	CTGCCCTGGG	ATTTCGCGAC	2520
GGCAGCTATT	GTGCGGAGAG	CATTATCGGC	ACCTGCGCGC	TGTCGCTGGC	CGCGATGCAG	2580
GGCCAGCCGA	TCAACACCGC	CGGCGATCGG	CATTTTAAGC	AGGCGCTACA	GCCATGGAGT	2640
TTTTGCTCGA	CGCCGGTGTT	TGATAACCAC	GGGCGGCTGT	TCGGCTCTAT	CTCGCTTTGC	2700
TGTCTGGTCG	AGCACCAGTC	CAGCGCCGAC	CTCTCCCTGA	CGCTGGCCAT	CGCCCGCGAG	2760
GTGGGTAACT	CCCTGCTTAC	CGACAGCCTG	CTGGCGGAAT	CCAACCGTCA	CCTCAATCAG	2820
ATGTACGGCC	TGCTGGAGAG	CATGGACGAT	GGGGTGATGG	CGTGGAACGA	ACAGGGCGTG	2880
CTGCAGTTTC	TCAATGTTCA	GGCGGCGAGA	CTGCTGCATC	TTGATGCTCA	GGCCAGCCAG	2940
GGGAAAAATA	TCGCCGATCT	GGTGACCCTC	CCGGCGCTGC	TGCGCCGCGC	CATCAAACAC	3000
GGCCGCGGCC	TGAATCACGT	CGAAGTCACC	TTTGAAAGTC	AGCATCAGTT	TGTCGATGCG	3060
GTGATCACCT	TAAAACCGAT	TGTCGAGGCG	CAAGGCAACA	GTTTTATTCT	GCTGCTGCAT	3120
CCGGTGGAGC	AGATGCGGCA	GCTGATGACC	AGCCAGCTCG	GTAAAGTCAG	CCACACCTTT	3180
GAGCAGATGT	CTGCCGACGA	TCCGGAAACC	CGACGCCTGA	TCCACTTTGG	CCGCCAGGCG	3240
GCGCGCGGCG	GCTTCCCGGT	GCTACTGTGC	GGCGAAGAGG	GGGTGCGGAA	AGAGCTGCTG	3300
AGCCAGGCTA	TTCACAATGA	AAGCGAACGG	GCGGGCGGCC	CCTACATCTC	CGTCAACTGC	3360
CAGCTATATG	CCGACAGCGT	GCTGGGCCAG	GACTTTATGG	GCAGCGCCCC	TACCGACGAT	3420
GAAAATGGTC	GCCTGAGCCG	CCTTGAGCTG	GCCAACGGCG	GCACCCTGTT	TCTGGAAAAG	3480
ATCGAGTATC	TGGCGCCGGA	GCTGCAGTCG	GCTCTGCTGC	AGGTGATTAA	GCAGGGCGTG	3540
CTCACCCGCC	TCGACGCCCG	GCGCCTGATC	CCGGTGGATG	TGAAGGTGAT	TGCCACCACC	3600
ACCGTCGATC	TGGCCAATCT	GGTGGAACAG	AACCGCTTTA	GCCGCCAGCT	GTACTATGCG	3660

CTGCACTCCT	TTGAGATCGT	CATCCCGCCG	CTGCGCGCCC	GACGCAACAG	TATTCCGTCTG	3720
CTGGTGCATA	ACCGGTTGAA	GAGCCTGGAG	AAGCGTTTCT	CTTCGCGACT	GAAAGTGGAC	3780
GATGACGCGC	TGGCACAGCT	GGTGGCCTAC	TCGTGGCCGG	GGAATGATTT	TGAGCTCAAC	3840
AGCGTCATTG	AGAATATCGC	CATCAGCAGC	GACAACGGCC	ACATTGCGCT	GAGTAATCTG	3900
CCGGAATATC	TCTTTTCCGA	GCGGCCGGGC	GGGGATAGCG	CGTCATCGCT	GCTGCCGGCC	3960
AGCCTGACTT	TTAGCGCCAT	CGAAAAGGAA	GCTATTATTC	ACGCCGCCCG	GGTGACCAGC	4020
GGGCGGGTGC	AGGAGATGTC	GCAGCTGCTC	AATATCGGCC	GCACCACCCT	GTGGCGCAAA	4080
ATGAAGCAGT	ACGATATTGA	CGCCAGCCAG	TTCAAGCGCA	AGCATCAGGC	CTAGTCTCTT	4140
CGATTGCGCG	CATGGAGAAC	AGGGCATCCG	ACAGGCGATT	GCTGTAGCGT	TTGAGCGCGT	4200
CGCGCAGCGG	ATGCGCGCGG	TCCATGGCCG	TCAGCAGGCG	TTGAGCCGA	CGGGACTGGG	4260
TGCGCGCCAC	GTGCAGCTGG	GCAGAGGCGA	GATTCTCTCC	CGGGATCACG	AACTGTTTTA	4320
ACGGGCCGCT	CTCGGCCATA	TTGCGGTCTG	TAAGCCGCTC	CAGGGCGGTG	ATCTCCTCTT	4380
CGCCGATCGT	CTGGCTCAGG	CGGGTCAGGC	CCCGCGCATC	GCTGGCCAGT	TCAGCCCCCA	4440
GCACGAACAG	CGTCTGCTGA	ATATGGTGCA	GGCTTTCCCG	CAGCCCGGCG	TCGCGGGTCG	4500
TGGCGTAGCA	GACGCCCAGC	TGGGATATCA	GTTTCATCGAC	GGTGCCGTAG	GCCTCGACGC	4560
GAATATGGTC	TTTCTCGATG	CGGCTGCCGC	CGTACAGGGC	GGTGGTGCCT	TTATCCCCGG	4620
TGCGGGTATA	GATACGATAC	ATTCAGTTTC	TCTCACTTAA	CGGCAGGACT	TTAACCAGCT	4680
GCCCGCGGTT	GGCGCCGAGC	GTACGCAGTT	GATCGTCGCT	ATCGGTGACG	TGTCCGGTAG	4740
CCAGCGGCGC	GTCCGCCGGC	AGCTGGGCAT	GAGTGAGGGC	TATCTCGCCG	GACGCGCTGA	4800
GCCCGATACC	CACCCGCAGG	GGCGAGCTTC	TGGCCGCCAG	GGCGCCGAGC	GCAGCGGCGT	4860
CACCGCCTCC	GTCATAGGTT	ATGGTCTGGC	AGGGGACCCC	CTGCTCCTCC	AGCCCCCAGC	4920
ACAGCTCATT	GATGGCGCCG	GCATGGTGCC	CGCGCGGATC	GTAACACAGG	CGTACGCCTG	4980
GCGGTGAAAG	CGACATGACG	GTCCCCTCGT	TAACACTCAG	AATGCCTGGC	GGAAAATCGC	5040
GGCAATCTCC	TGCTCGTTGC	CTTTACGCGG	GTTTCGAGAAC	GCATTGCCGT	CTTTTAGAGC	5100
CATCTCCGCC	ATGTAGGGGA	AGTCGGCCTC	TTTTACCCCC	AGATCGCGCA	GATGCTGCGG	5160
AATACCGATA	TCCATCGACA	GACGCGTGAT	AGCGGCGATG	GCTTTTTCCG	CCGCGTCGAG	5220
AGTGGACAGT	CCGGTGATAT	TTTCGCCCAT	CAGTTCAGCG	ATATCGGCGA	ATTTCTCCGG	5280
GTTGGCGATC	AGGTTGTAGC	GCGCCACATG	CGGCAGCAGG	ACAGCGTTGG	CCACGCCGTG	5340
CGGCATGTCG	TACAGGCCGC	CCAGCTGGTG	CGCCATGGCG	TGCACGTAGC	CGAGGTTGGC	5400
GTTATTGAAA	GCCATCCCGG	CCAGCAGAGA	AGCATAGGCC	ATGTTTTCCC	GCGCCTGCAG	5460
ATTGCTGCCG	AGGGCCACGG	CCTGGCGCAG	GTTGCGGGCG	ATGAGGCGGA	TCGCCTGCAT	5520
GGCGGCGGCG	TCCGTCACCG	GGTTAGCGTC	TTTGAGATA	TAGGCCTCTA	CGGCGTGGGT	5580
CAGGGCATCC	ATCCCGGTCTG	CCGCGGTCTG	GGCGGCCGGT	TTACCGATCA	TCAGCAGTGG	5640



ATCGTTGATA GAGACCGACG GCAGTTTGCG CCAGCTGACG ATCACAAACT TCACTTTGGT	5700
TTCGGTGTG GTCAGGACGC AGTGGCGGGT GACCTCGCTG GCGGTGCCGG CCGTGGTATT	5760
GACCGCGACG ATAGGCGGCA GCGGGTTGGT CAGGGTCTCG ATTCCGGCAT ACTGGTACAG	5820
ATCGCCCTCA TGGGTGGCGG CGATGCCGAT GCCTTTGCCG CAATCGTGCG GGCTGCCGCC	5880
GCCCACGGTG ACGATGATGT CGCACTGTTC GCGGCGAAAC ACGGCGAGGC CGTCGCGCAC	5940
GTTGGTGTCT TTCGGGTTCG GCTCGACGCC GTCAAAGATC GCCACCTCGA TCCCGGCCTC	6000
CCGCAGATAA TGCAGGGTTT TGTCCACCGC GCCATCTTTA ATTGCCCCGA GGCCTTTGTC	6060
GGTGACCAGC AGGGCTTTTT TCCCCCCCAG CAGCTGGCAG CGTTCGCCGA CTACGGAAAT	6120
GGCGTTGGGG CCAAAAAAGT TAACGTTTGG CACCAGATAA TCAAACATAC GATAGCTCAT	6180
AATATACCTT CTCGCTTCAG GTTATAATGC GGAAAAACAA TCCAGGGCGC ACTGGGCTAA	6240
TAATTGATCC TGCTCGACCG TACCGCCGCT AACGCCGACG GCGCCAATTA CCTGCTCATT	6300
AAAAATAACT GGCAGGCCGC CGCCAAAAAT AATAATTTCG TGTGGTTGG TTAGCTGCAG	6360
ACCGTACAGA GATTGTCCTG GCTGGACCGC TGACGTAATT TCATGGGTAC CTTGCTTCAG	6420
GCTGCAGGCG CTCCAGGCTT TATTCAGGGA AATATCGCAG CTGGAGACGA AGGCCTCGTC	6480
CATCCGCTGG ATAAGCAGCG TGTTCCTCC GCGGTCAACT ACGGAAACA CCACGCCAC	6540
GTTGATCTCA GTGGCTTTTT TTTCCACCGC CGCCGCCATT TGCTGGGCGG CGGCCAGGGT	6600
GATTGTCTGA ACTTGTGGC TCTTGTTTTCAT CATTCTCTCC CGCACCAGGA TAACGCTGGC	6660
GCGAATAGTC AGTAGGGGGC GATAGTAAAA AACTATTACC ATTCGGTTGG CTTGCTTTAT	6720
TTTTGTCAGC GTTATTTTGT CGCCCGCCAT GATTTAGTCA ATAGGGTTAA AATAGCGTCG	6780
GAAAAACGTA ATTAAGGGCG TTTTTTATTA ATTGATTTAT ATCATTGCGG GCGATCACAT	6840
TTTTTATTTT TGCCGCCGGA GTAAAGTTTC ATAGTGAAAC TGTCGGTAGA TTTCTGTGC	6900
CAAATTGAAA CGAAATTAAA TTTATTTTTT TCACCACTGG CTCATTTAAA GTTCCGCTAT	6960
TGCCGGTAAT GGCCGGGCGG CAACGACGCT GGCCCGGCGT ATTCGCTACC GTCTGCGGAT	7020
TTCACCTTTT GAGCCGATGA ACAATGAAAA GATCAAAACG ATTTGCAGTA CTGGCCCAGC	7080
GCCCCGTCAA TCAGGACGGG CTGATTGGCG AGTGGCCTGA AGAGGGGCTG ATCGCCATGG	7140
ACAGCCCCTT TGACCCGGTC TCTTCAGTAA AAGTGGACAA CGGTCTGATC GTCGAACTGG	7200
ACGGCAAACG CCGGGACCAG TTTGACATGA TCGACCGATT TATCGCCGAT TACGCGATCA	7260
ACGTTGAGCG CACAGAGCAG GCAATGCGCC TGGAGGCGGT GGAAATAGCC CGTATGCTGG	7320
TGGATATTCA CGTCAGCCGG GAGGAGATCA TTGCCATCAC TACCGCCATC ACGCCGGCCA	7380
AAGCGGTCGA GGTGATGGCG CAGATGAACG TGGTGGAGAT GATGATGGCG CTGCAGAAGA	7440
TGCGTGCCCG CCGGACCCCC TCCAACCAGT GCCACGTCAC CAATCTCAAA GATAATCCGG	7500
TGCAGATTGC CGCTGACGCC GCCGAGGCCG GGATCCGCGG CTTCTCAGAA CAGGAGACCA	7560
CGGTCCGTAT CGCGCGCTAC GCGCCGTTTA ACGCCCTGGC GCTGTTGGTC GGTTCGCAGT	7620

GCGGCCGCCC CGGCGTGTG ACGCAGTGCT CGGTGGAAGA GGCCACCGAG CTGGAGCTGG	7680
GCATGCGTGG CTTAACCAGC TACGCCGAGA CGGTGTCGGT CTACGGCACC GAAGCGGTAT	7740
TTACCGACGG CGATGATACG CCGTGGTCAA AGGCGTTCCT CGCCTCGGCC TACGCCTCCC	7800
GCGGGTTGAA AATGCGCTAC ACCTCCGGCA CCGGATCCGA AGCGCTGATG GGCTATTCTGG	7860
AGAGCAAGTC GATGCTCTAC CTCGAATCGC GCTGCATCTT CATTACTAAA GGCGCCGGGG	7920
TTCAGGGACT GCAAAACGGC GCGGTGAGCT GTATCGGCAT GACCGGCGCT GTGCCGTCGG	7980
GCATTGCGGC GGTGCTGGCG GAAAACCTGA TCGCCTCTAT GCTCGACCTC GAAGTGGCGT	8040
CCGCCAACGA CCAGACTTTC TCCCACTCGG ATATTGCGCG CACCGCGCGC ACCCTGATGC	8100
AGATGCTGCC GGGCACCGAC TTTATTTTCT CCGGCTACAG CGCGGTGCCG AACTACGACA	8160
ACATGTTTCG CGGCTCGAAC TTCGATGCGG AAGATTTTGA TGATTACAAC ATCCTGCAGC	8220
GTGACCTGAT GGTGACGGC GGCCTGCGTC CGGTGACCGA GGCGGAAACC ATTGCCATT	8280
GCCAGAAAGC GGCGCGGGCG ATCCAGGCGG TTTTCCGCGA GCTGGGGCTG CCGCCAATCG	8340
CCGACGAGGA GGTGGAGGCC GCCACCTACG CGCACGGCAG CAACGAGATG CCGCCGCGTA	8400
ACGTGGTGGG GGATCTGAGT GCGGTGGAAG AGATGATGAA GCGCAACATC ACCGGCCTCG	8460
ATATTGTCGG CGCGCTGAGC CGCAGCGGCT TTGAGGATAT CGCCAGCAAT ATTCTCAATA	8520
TGCTGCGCCA GCGGGTCACC GCGGATTACC TGCAGACCTC GGCCATTCTC GATCGGCAGT	8580
TCGAGGTGGT GAGTGCGGTC AACGACATCA ATGACTATCA GGGGCCGGGC ACCGGCTATC	8640
GCATCTCTGC CGAACGCTGG GCGGAGATCA AAAATATTCC GGGCGTGGTT CAGCCCAGACA	8700
CCATTGAATA AGGCGGTATT CCTGTGCAAC AGACAACCCA AATTCAGCCC TCTTTTACCC	8760
TGAAAACCCG CGAGGGCGGG GTAGCTTCTG CCGATGAACG CGCCGATGAA GTGGTGATCG	8820
GCGTCGGCCC TGCCTTCGAT AAACACCAGC ATCACACTCT GATCGATATG CCCCATGGCG	8880
CGATCCTCAA AGAGCTGATT GCCGGGGTGG AAGAAGAGGG GCTTCACGCC CGGGTGGTGC	8940
GCATTCTGCG CACGTCCGAC GTCTCCTTTA TGGCCTGGGA TGCGGCCAAC CTGAGCGGCT	9000
CGGGGATCGG CATCGGTATC CAGTCGAAGG GGACCACGGT CATCCATCAG CGCGATCTGC	9060
TGCCGCTCAG CAACCTGGAG CTGTTCTCCC AGGCGCCGCT GCTGACGCTG GAGACCTACC	9120
GGCAGATTGG CAAAACGCT GCGCGCTATG CGCGCAAAGA GTCACCTTCG CCGGTGCCGG	9180
TGGTGAACGA TCAGATGGTG CGGCCGAAAT TTATGGCCAA AGCCGCGCTA TTTCATATCA	9240
AAGAGACCAA ACATGTGGTG CAGGACGCCG AGCCCGTCAC CCTGCACATC GACTTAGTAA	9300
GGGAGTGACC ATGAGCGAGA AAACCATGCG CGTGCAGGAT TATCCGTTAG CCACCCGCTG	9360
CCCGGAGCAT ATCTGACGC CTACCGGCAA ACCATTGACC GATATTACCC TCGAGAAGGT	9420
GCTCTCTGGC GAGGTGGGCC CGCAGGATGT GCGGATCTCC CGCCAGACCC TTGAGTACCA	9480
GGCGCAGATT GCCGAGCAGA TGCAGCGCCA TGCGGTGGCG CGCAATTTCG GCCGCGCGGC	9540
GGAGCTTATC GCCATTCTTG ACGAGCGCAT TCTGGCTATC TATAACGCGC TGCGCCCGTT	9600

CCGCTCCTCG	CAGGCGGAGC	TGCTGGCGAT	CGCCGACGAG	CTGGAGCACA	CCTGGCATGC	9660
GACAGTGAAT	GCCGCCTTTG	TCCGGGAGTC	GGCGGAAGTG	TATCAGCAGC	GGCATAAGCT	9720
GCGTAAAGGA	AGCTAAGCGG	AGGTCAGCAT	GCCGTTAATA	GCCGGGATTG	ATATCGGCAA	9780
CGCCACCACC	GAGGTGGCGC	TGGCGTCCGA	CTACCCGCAG	GCGAGGGCGT	TTGTTGCCAG	9840
CGGGATCGTC	GCGACGACGG	GCATGAAAGG	GACGCGGGAC	AATATCGCCG	GGACCCTCGC	9900
CGCGCTGGAG	CAGGCCCTGG	CGAAAACACC	GTGGTCGATG	AGCGATGTCT	CTCGCATCTA	9960
TCTTAACGAA	GCCGCGCCGG	TGATTGGCGA	TGTGGCGATG	GAGACCATCA	CCGAGACCAT	10020
TATCACCGAA	TCGACCATGA	TCGGTCATAA	CCCGCAGACG	CCGGGCGGGG	TGGGCGTTGG	10080
CGTGGGGACG	ACTATCGCCC	TCGGGCGGGT	GGCGACGCTG	CCGGCGGGCG	AGTATGCCGA	10140
GGGGTGGATC	GTACTGATTG	ACGACGCCGT	CGATTTCTTT	GACGCCGTGT	GGTGGCTCAA	10200
TGAGGCGCTC	GACCGGGGGA	TCAACGTGGT	GGCGGCGATC	CTCAAAAAGG	ACGACGGCGT	10260
GCTGGTGAAC	AACCGCCTGC	GTAAAACCCT	GCCGGTGGTG	GATGAAGTGA	CGCTGCTGGA	10320
GCAGGTCCCC	GAGGGGGTAA	TGGCGGCGGT	GGAAGTGGCC	GCGCCGGGCC	AGGTGGTGCG	10380
GATCCTGTGC	AATCCCTACG	GGATCGCCAC	CTTCTTCGGG	CTAAGCCCGG	AAGAGACCCA	10440
GGCCATCGTC	CCCATCGCCC	GCGCCCTGAT	TGGCAACCGT	TCCGCGGTGG	TGCTCAAGAC	10500
CCCGCAGGGG	GATGTGCAGT	CGCGGGTGAT	CCCGGCGGGC	AACCTCTACA	TTAGCGGCGA	10560
AAAGCGCCGC	GGAGAGGCCG	ATGTCGCCGA	GGGCGCGGAA	GCCATCATGC	AGGCGATGAG	10620
CGCCTGCGCT	CCGGTACGCG	ACATCCGCGG	CGAACCGGGC	ACCCACGCCG	GCGGCATGCT	10680
TGAGCGGGTG	CGCAAGGTAA	TGGCGTCCCT	GACCGGCCAT	GAGATGAGCG	CGATATACAT	10740
CCAGGATCTG	CTGGCGGTGG	ATACGTTTAT	TCCGCGCAAG	GTGCAGGGCG	GGATGGCCGG	10800
CGAGTGCGCC	ATGGAGAATG	CCGTCGGGAT	GGCGGCGATG	GTGAAAGCGG	ATCGTCTGCA	10860
AATGCAGGTT	ATCGCCCGCG	AACTGAGCGC	CCGACTGCAG	ACCGAGGTGG	TGGTGGGCGG	10920
CGTGAGGGCC	AACATGGCCA	TCGCCGGGGC	GTTAACCCTT	CCCGGCTGTG	CGGCGCCGCT	10980
GGCGATCCTC	GACCTCGGCG	CCGGCTCGAC	GGATGCGGCG	ATCGTCAACG	CGGAGGGGCA	11040
GATAACGGCG	GTCCATCTCG	CCGGGGCGGG	GAATATGGTC	AGCCTGTTGA	TTAAAACCGA	11100
GCTGGGCCTC	GAGGATCTTT	CGCTGGCGGA	AGCGATAAAA	AAATACCCGC	TGGCCAAAGT	11160
GGAAAGCCTG	TTCAGTATTC	GTCACGAGAA	TGGCGCGGTG	GAGTTCTTTC	GGGAAGCCCT	11220
CAGCCCGGCG	GTGTTCGCCA	AAGTGGTGTA	CATCAAGGAG	GGCGAACTGG	TGCCGATCGA	11280
TAACGCCAGC	CCGCTGGAAA	AAATTCGTCT	CGTGCGCCGG	CAGGCGAAAG	AGAAAGTGTT	11340
TGTCACCAAC	TGCCTGCGCG	CGCTGCGCCA	GGTCTCACCC	GGCGGTTCCA	TTGCGGATAT	11400
CGCCTTTGTG	GTGCTGGTGG	GCGGCTCATC	GCTGGACTTT	GAGATCCCGC	AGCTTATCAC	11460
GGAAGCCTTG	TCGCACTATG	GCGTGGTCGC	CGGGCAGGGC	AATATTCGGG	GAACAGAAGG	11520
GCCGCGCAAT	GCGGTGCGCA	CCGGGCTGCT	ACTGGCCGGT	CAGGCGAATT	AAACGGGCGC	11580

TCGCGCCAGC CTCTCTCTTT AACGTGCTAT TTCAGGATGC CGATAATGAA CCAGACTTCT 11640  
 ACCTTAACCG GGCAGTGC GT GGCCGAGTTT CTTGGCACC GATTGCTCAT TTTCTTCGGC 11700  
 GCGGGCTGCG TCGCTGCGCT GCGGGTCGCC GGGGCCAGCT TTGGTCAGTG GGAGATCAGT 11760  
 ATTATCTGGG GCCTTGGCGT CGCCATGGCC ATCTACCTGA CGGCCGGTGT CTCCGGCGCG 11820  
 CACCTAAATC CGGCGGTGAC CATTGCCCTG TGGCTGTTTCG CCTGTTTTGA ACGCCGCAAG 11880  
 GTGCTGCCGT TTATTGTTGC CCAGACGGCC GGGGCCTTCT GCGCCGCCGC GCTGGTGTAT 11940  
 GGGCTCTATC GCCAGCTGTT TCTCGATCTT GAACAGAGTC AGCATATCGT GCGCGGCACT 12000  
 GCCGCCAGTC TTAACCTGGC CGGGGTCTTT TCCACGTACC CGCATCCACA TATCACTTTT 12060  
 ATACAAGCGT TTGCCGTGGA GACCACCATC ACGGCAATCC TGATGGCGAT GATCATGGCC 12120  
 CTGACCGACG ACGGCAACGG AATTC 12145

## (2) INFORMATION FOR SEQ ID NO:20:

- (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 94 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

AGCTTAGGAG TCTAGAATAT TGAGCTCGAA TTCCCGGGCA TGCGGTACCG GATCCAGAAA 60  
 AAAGCCCGCA CCTGACAGTG CGGGCTTTTT TTTT 94

## (2) INFORMATION FOR SEQ ID NO:21:

- (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 37 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

GGAATTCAGA TCTCAGCAAT GAGCGAGAAA ACCATGC 37

## (2) INFORMATION FOR SEQ ID NO:22:

- (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 27 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

GCTCTAGATT AGCTTCCTTT ACGCAGC 27

## (2) INFORMATION FOR SEQ ID NO:23:

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 33 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: DNA (genomic)
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:  
GGCCAAGCTT AAGGAGGTTA ATTAAATGAA AAG 33
- (2) INFORMATION FOR SEQ ID NO:24:
- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 26 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: DNA (genomic)
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:  
GCTCTAGATT ATTCAATGGT GTCGGG 26
- (2) INFORMATION FOR SEQ ID NO:25:
- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 42 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: DNA (genomic)
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:  
GCGCCGTCTA GAATTATGAG CTATCGTATG TTTGATTATC TG 42
- (2) INFORMATION FOR SEQ ID NO:26:
- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 36 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: DNA (genomic)
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:26:  
TCTGATACGG GATCCTCAGA ATGCCTGGCG GAAAAT 36
- (2) INFORMATION FOR SEQ ID NO:27:
- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 51 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:27:  
GCGCGGATCC AGGAGTCTAG AATTATGGGA TTGACTACTA AACCTCTATC T 51

(2) INFORMATION FOR SEQ ID NO:28:

(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 36 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:28:  
GATACGCCCCG GGTTACCATT TCAACAGATC GTCCTT 36

(2) INFORMATION FOR SEQ ID NO:29:

(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 18 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:29:  
TCGACGAATT CAGGAGGA 18

(2) INFORMATION FOR SEQ ID NO:30:

(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 18 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:30:  
CTAGTCCTCC TGAATTCG 18

(2) INFORMATION FOR SEQ ID NO:31:

(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 19 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:31:  
CTAGTAAGGA GGACAATTC 19

(2) INFORMATION FOR SEQ ID NO:32:

(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 19 base pairs  
(B) TYPE: nucleic acid

(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:32:

CATGGAATTG TCCTCCTTA

19

(2) INFORMATION FOR SEQ ID NO:33:

(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 271 amino acids  
(B) TYPE: amino acid  
(C) STRANDEDNESS: unknown  
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: protein

(vi) ORIGINAL SOURCE:  
(A) ORGANISM: GPPI

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:33:

Met	Lys	Arg	Phe	Asn	Val	Leu	Lys	Tyr	Ile	Arg	Thr	Thr	Lys	Ala	Asn	1	5	10	15
Ile	Gln	Thr	Ile	Ala	Met	Pro	Leu	Thr	Thr	Lys	Pro	Leu	Ser	Leu	Lys	20	25	30	
Ile	Asn	Ala	Ala	Leu	Phe	Asp	Val	Asp	Gly	Thr	Ile	Ile	Ile	Ser	Gln	35	40	45	
Pro	Ala	Ile	Ala	Ala	Phe	Trp	Arg	Asp	Phe	Gly	Lys	Asp	Lys	Pro	Tyr	50	55	60	
Phe	Asp	Ala	Glu	His	Val	Ile	His	Ile	Ser	His	Gly	Trp	Arg	Thr	Tyr	65	70	75	80
Asp	Ala	Ile	Ala	Lys	Phe	Ala	Pro	Asp	Phe	Ala	Asp	Glu	Glu	Tyr	Val	85	90	95	
Asn	Lys	Leu	Glu	Gly	Glu	Ile	Pro	Glu	Lys	Tyr	Gly	Glu	His	Ser	Ile	100	105	110	
Glu	Val	Pro	Gly	Ala	Val	Lys	Leu	Cys	Asn	Ala	Leu	Asn	Ala	Leu	Pro	115	120	125	
Lys	Glu	Lys	Trp	Ala	Val	Ala	Thr	Ser	Gly	Thr	Arg	Asp	Met	Ala	Lys	130	135	140	
Lys	Trp	Phe	Asp	Ile	Leu	Lys	Ile	Lys	Arg	Pro	Glu	Tyr	Phe	Ile	Thr	145	150	155	160
Ala	Asn	Asp	Val	Lys	Gln	Gly	Lys	Pro	His	Pro	Glu	Pro	Tyr	Leu	Lys	165	170	175	
Gly	Arg	Asn	Gly	Leu	Gly	Phe	Pro	Ile	Asn	Glu	Gln	Asp	Pro	Ser	Lys	180	185	190	
Ser	Lys	Val	Val	Val	Phe	Glu	Asp	Ala	Pro	Ala	Gly	Ile	Ala	Ala	Gly	195	200	205	
Lys	Ala	Ala	Gly	Cys	Lys	Ile	Val	Gly	Ile	Ala	Thr	Thr	Phe	Asp	Leu	210	215	220	

Asp Phe Leu Lys Glu Lys Gly Cys Asp Ile Ile Val Lys Asn His Glu  
 225 230 235 240  
 Ser Ile Arg Val Gly Glu Tyr Asn Ala Glu Thr Asp Glu Val Glu Leu  
 245 250 255  
 Ile Phe Asp Asp Tyr Leu Tyr Ala Lys Asp Asp Leu Leu Lys Trp  
 260 265 270

## (2) INFORMATION FOR SEQ ID NO:34:

- (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 555 amino acids  
 (B) TYPE: amino acid  
 (C) STRANDEDNESS: unknown  
 (D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: protein

(vi) ORIGINAL SOURCE:  
 (A) ORGANISM: DHAB1

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:34:

Met Lys Arg Ser Lys Arg Phe Ala Val Leu Ala Gln Arg Pro Val Asn  
 1 5 10 15  
 Gln Asp Gly Leu Ile Gly Glu Trp Pro Glu Glu Gly Leu Ile Ala Met  
 20 25 30  
 Asp Ser Pro Phe Asp Pro Val Ser Ser Val Lys Val Asp Asn Gly Leu  
 35 40 45  
 Ile Val Glu Leu Asp Gly Lys Arg Arg Asp Gln Phe Asp Met Ile Asp  
 50 55 60  
 Arg Phe Ile Ala Asp Tyr Ala Ile Asn Val Glu Arg Thr Glu Gln Ala  
 65 70 75 80  
 Met Arg Leu Glu Ala Val Glu Ile Ala Arg Met Leu Val Asp Ile His  
 85 90 95  
 Val Ser Arg Glu Glu Ile Ile Ala Ile Thr Thr Ala Ile Thr Pro Ala  
 100 105 110  
 Lys Ala Val Glu Val Met Ala Gln Met Asn Val Val Glu Met Met Met  
 115 120 125  
 Ala Leu Gln Lys Met Arg Ala Arg Arg Thr Pro Ser Asn Gln Cys His  
 130 135 140  
 Val Thr Asn Leu Lys Asp Asn Pro Val Gln Ile Ala Ala Asp Ala Ala  
 145 150 155 160  
 Glu Ala Gly Ile Arg Gly Phe Ser Glu Gln Glu Thr Thr Val Gly Ile  
 165 170 175  
 Ala Arg Tyr Ala Pro Phe Asn Ala Leu Ala Leu Leu Val Gly Ser Gln  
 180 185 190  
 Cys Gly Arg Pro Gly Val Leu Thr Gln Cys Ser Val Glu Glu Ala Thr  
 195 200 205  
 Glu Leu Glu Leu Gly Met Arg Gly Leu Thr Ser Tyr Ala Glu Thr Val  
 210 215 220



Ser Val Tyr Gly Thr Glu Ala Val Phe Thr Asp Gly Asp Asp Thr Pro  
 225 230 235 240  
 Trp Ser Lys Ala Phe Leu Ala Ser Ala Tyr Ala Ser Arg Gly Leu Lys  
 245 250 255  
 Met Arg Tyr Thr Ser Gly Thr Gly Ser Glu Ala Leu Met Gly Tyr Ser  
 260 265 270  
 Glu Ser Lys Ser Met Leu Tyr Leu Glu Ser Arg Cys Ile Phe Ile Thr  
 275 280 285  
 Lys Gly Ala Gly Val Gln Gly Leu Gln Asn Gly Ala Val Ser Cys Ile  
 290 295 300  
 Gly Met Thr Gly Ala Val Pro Ser Gly Ile Arg Ala Val Leu Ala Glu  
 305 310 315 320  
 Asn Leu Ile Ala Ser Met Leu Asp Leu Glu Val Ala Ser Ala Asn Asp  
 325 330 335  
 Gln Thr Phe Ser His Ser Asp Ile Arg Arg Thr Ala Arg Thr Leu Met  
 340 345 350  
 Gln Met Leu Pro Gly Thr Asp Phe Ile Phe Ser Gly Tyr Ser Ala Val  
 355 360 365  
 Pro Asn Tyr Asp Asn Met Phe Ala Gly Ser Asn Phe Asp Ala Glu Asp  
 370 375 380  
 Phe Asp Asp Tyr Asn Ile Leu Gln Arg Asp Leu Met Val Asp Gly Gly  
 385 390 395 400  
 Leu Arg Pro Val Thr Glu Ala Glu Thr Ile Ala Ile Arg Gln Lys Ala  
 405 410 415  
 Ala Arg Ala Ile Gln Ala Val Phe Arg Glu Leu Gly Leu Pro Pro Ile  
 420 425 430  
 Ala Asp Glu Glu Val Glu Ala Ala Thr Tyr Ala His Gly Ser Asn Glu  
 435 440 445  
 Met Pro Pro Arg Asn Val Val Glu Asp Leu Ser Ala Val Glu Glu Met  
 450 455 460  
 Met Lys Arg Asn Ile Thr Gly Leu Asp Ile Val Gly Ala Leu Ser Arg  
 465 470 475 480  
 Ser Gly Phe Glu Asp Ile Ala Ser Asn Ile Leu Asn Met Leu Arg Gln  
 485 490 495  
 Arg Val Thr Gly Asp Tyr Leu Gln Thr Ser Ala Ile Leu Asp Arg Gln  
 500 505 510  
 Phe Glu Val Val Ser Ala Val Asn Asp Ile Asn Asp Tyr Gln Gly Pro  
 515 520 525  
 Gly Thr Gly Tyr Arg Ile Ser Ala Glu Arg Trp Ala Glu Ile Lys Asn  
 530 535 540  
 Ile Pro Gly Val Val Gln Pro Asp Thr Ile Glu  
 545 550 555

## (2) INFORMATION FOR SEQ ID NO:35:

- (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 194 amino acids  
 (B) TYPE: amino acid  
 (C) STRANDEDNESS: unknown  
 (D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: protein

(vi) ORIGINAL SOURCE:  
 (A) ORGANISM: DHAB2

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:35:

```

Met Gln Gln Thr Thr Gln Ile Gln Pro Ser Phe Thr Leu Lys Thr Arg
1          5          10          15
Glu Gly Gly Val Ala Ser Ala Asp Glu Arg Ala Asp Glu Val Val Ile
20          25          30
Gly Val Gly Pro Ala Phe Asp Lys His Gln His His Thr Leu Ile Asp
35          40          45
Met Pro His Gly Ala Ile Leu Lys Glu Leu Ile Ala Gly Val Glu Glu
50          55          60
Glu Gly Leu His Ala Arg Val Val Arg Ile Leu Arg Thr Ser Asp Val
65          70          75          80
Ser Phe Met Ala Trp Asp Ala Ala Asn Leu Ser Gly Ser Gly Ile Gly
85          90          95
Ile Gly Ile Gln Ser Lys Gly Thr Thr Val Ile His Gln Arg Asp Leu
100         105         110
Leu Pro Leu Ser Asn Leu Glu Leu Phe Ser Gln Ala Pro Leu Leu Thr
115         120         125
Leu Glu Thr Tyr Arg Gln Ile Gly Lys Asn Ala Ala Arg Tyr Ala Arg
130         135         140
Lys Glu Ser Pro Ser Pro Val Pro Val Val Asn Asp Gln Met Val Arg
145         150         155         160
Pro Lys Phe Met Ala Lys Ala Ala Leu Phe His Ile Lys Glu Thr Lys
165         170         175
His Val Val Gln Asp Ala Glu Pro Val Thr Leu His Ile Asp Leu Val
180         185         190
Arg Glu

```

## (2) INFORMATION FOR SEQ ID NO:36:

- (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 140 amino acids  
 (B) TYPE: amino acid  
 (C) STRANDEDNESS: unknown  
 (D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: protein

(vi) ORIGINAL SOURCE:  
 (A) ORGANISM: DHAB3

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:36:

Met Ser Glu Lys Thr Met Arg Val Gln Asp Tyr Pro Leu Ala Thr Arg  
 1 5 10 15  
 Cys Pro Glu His Ile Leu Thr Pro Thr Gly Lys Pro Leu Thr Asp Ile  
 20 25 30  
 Thr Leu Glu Lys Val Leu Ser Gly Glu Val Gly Pro Gln Asp Val Arg  
 35 40 45  
 Ile Ser Arg Gln Thr Leu Glu Tyr Gln Ala Gln Ile Ala Glu Gln Met  
 50 55 60  
 Gln His Ala Val Ala Arg Asn Phe Arg Arg Ala Ala Glu Leu Ile Ala  
 65 70 75 80  
 Ile Pro Asp Glu Arg Ile Leu Ala Ile Tyr Asn Ala Leu Arg Pro Phe  
 85 90 95  
 Arg Ser Ser Gln Ala Glu Leu Leu Ala Ile Ala Asp Glu Leu Glu His  
 100 105 110  
 Thr Trp His Ala Thr Val Asn Ala Ala Phe Val Arg Glu Ser Ala Glu  
 115 120 125  
 Val Tyr Gln Gln Arg His Lys Leu Arg Lys Gly Ser  
 130 135 140

## (2) INFORMATION FOR SEQ ID NO:37:

- (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 387 amino acids  
 (B) TYPE: amino acid  
 (C) STRANDEDNESS: unknown  
 (D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: protein

(vi) ORIGINAL SOURCE:  
 (A) ORGANISM: DHAT

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:37:

Met Ser Tyr Arg Met Phe Asp Tyr Leu Val Pro Asn Val Asn Phe Phe  
 1 5 10 15  
 Gly Pro Asn Ala Ile Ser Val Val Gly Glu Arg Cys Gln Leu Leu Gly  
 20 25 30  
 Gly Lys Lys Ala Leu Leu Val Thr Asp Lys Gly Leu Arg Ala Ile Lys  
 35 40 45  
 Asp Gly Ala Val Asp Lys Thr Leu His Tyr Leu Arg Glu Ala Gly Ile  
 50 55 60  
 Glu Val Ala Ile Phe Asp Gly Val Glu Pro Asn Pro Lys Asp Thr Asn  
 65 70 75 80  
 Val Arg Asp Gly Leu Ala Val Phe Arg Arg Glu Gln Cys Asp Ile Ile  
 85 90 95  
 Val Thr Val Gly Gly Gly Ser Pro His Asp Cys Gly Lys Gly Ile Gly  
 100 105 110

Ile Ala Ala Thr His Glu Gly Asp Leu Tyr Gln Tyr Ala Gly Ile Glu  
 115 120 125  
 Thr Leu Thr Asn Pro Leu Pro Pro Ile Val Ala Val Asn Thr Thr Ala  
 130 135 140  
 Gly Thr Ala Ser Glu Val Thr Arg His Cys Val Leu Thr Asn Thr Glu  
 145 150 155 160  
 Thr Lys Val Lys Phe Val Ile Val Ser Trp Arg Lys Leu Pro Ser Val  
 165 170 175  
 Ser Ile Asn Asp Pro Leu Leu Met Ile Gly Lys Pro Ala Ala Leu Thr  
 180 185 190  
 Ala Ala Thr Gly Met Asp Ala Leu Thr His Ala Val Glu Ala Tyr Ile  
 195 200 205  
 Ser Lys Asp Ala Asn Pro Val Thr Asp Ala Ala Ala Met Gln Ala Ile  
 210 215 220  
 Arg Leu Ile Ala Arg Asn Leu Arg Gln Ala Val Ala Leu Gly Ser Asn  
 225 230 235 240  
 Leu Gln Ala Arg Glu Asn Met Ala Tyr Ala Ser Leu Leu Ala Gly Met  
 245 250 255  
 Ala Phe Asn Asn Ala Asn Leu Gly Tyr Val His Ala Met Ala His Gln  
 260 265 270  
 Leu Gly Gly Leu Tyr Asp Met Pro His Gly Val Ala Asn Ala Val Leu  
 275 280 285  
 Leu Pro His Val Ala Arg Tyr Asn Leu Ile Ala Asn Pro Glu Lys Phe  
 290 295 300  
 Ala Asp Ile Ala Glu Leu Met Gly Glu Asn Ile Thr Gly Leu Ser Thr  
 305 310 315 320  
 Leu Asp Ala Ala Glu Lys Ala Ile Ala Ala Ile Thr Arg Leu Ser Met  
 325 330 335  
 Asp Ile Gly Ile Pro Gln His Leu Arg Asp Leu Gly Val Lys Glu Ala  
 340 345 350  
 Asp Phe Pro Tyr Met Ala Glu Met Ala Leu Lys Asp Gly Asn Ala Phe  
 355 360 365  
 Ser Asn Pro Arg Lys Gly Asn Glu Gln Glu Ile Ala Ala Ile Phe Arg  
 370 375 380  
 Gln Ala Phe  
 385

## (2) INFORMATION FOR SEQ ID NO:38:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 27 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: DNA (genomic)

- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:38:  
GCGAATTCAT GAGCTATCGT ATGTTTG 27
- (2) INFORMATION FOR SEQ ID NO:39:
- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 28 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: DNA (genomic)
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:39:  
GCGAATTCAG AATGCCTGGC GGAAAATC 28
- (2) INFORMATION FOR SEQ ID NO:40:
- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 28 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: DNA (genomic)
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:40:  
GGGAATTCAT GAGCGAGAAA ACCATGCG 28
- (2) INFORMATION FOR SEQ ID NO:41:
- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 27 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: DNA (genomic)
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:41:  
GCGAATTCTT AGCTTCCTTT ACGCAGC 27
- (2) INFORMATION FOR SEQ ID NO:42:
- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 30 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: DNA (genomic)
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:42:  
GCGAATTCAT GCAACAGACA ACCCAAATTC 30
- (2) INFORMATION FOR SEQ ID NO:43:
- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 25 base pairs  
(B) TYPE: nucleic acid

(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:43:

GCGAATTCAC TCCCTTACTA AGTCG 25

(2) INFORMATION FOR SEQ ID NO:44:

(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 30 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:44:

GGAATTCAT GAAAAGATCA AAACGATTG 30

(2) INFORMATION FOR SEQ ID NO:45:

(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 29 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:45:

GCGAATTCTT ATTCAATGGT GTCGGGCTG 29

(2) INFORMATION FOR SEQ ID NO:46

(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 34 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:46:

TTGATAATAT AACCATGGCT GCTGCTGCTG ATAG 34

(2) INFORMATION FOR SEQ ID NO:47

(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 39 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:47:

GTATGATATG TTATCTTGGA TCCAATAAAT CTAATCTTC 39

## (2) INFORMATION FOR SEQ ID NO:48:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 24 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:48:

CATGACTAGT AAGGAGGACA ATTC

24

## (2) INFORMATION FOR SEQ ID NO:49:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 24 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:49:

CATGGAATTG TCCTCCTTAC TAGT

24

## INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

(PCT Rule 13*bts*)

<b>A.</b> The indications made below relate to the microorganism referred to in the description on page <u>7 and 8</u> , lines <u>37 &amp; 38 on pg. 7 &amp; Lines 1-5 on pg. 8</u>	
<b>B. IDENTIFICATION OF DEPOSIT</b> <span style="float: right;">Further deposits are identified on an additional sheet <input type="checkbox"/></span>	
Name of depositary institution <b>AMERICAN TYPE CULTURE COLLECTION</b>	
Address of depositary institution (including postal code and country) <b>12301 Parklawn Drive          Rockville, Maryland 20852          US</b>	
Date of deposit <b>26 September 1996</b>	Accession Number <b>98188</b>
<b>C. ADDITIONAL INDICATIONS</b> (leave blank if not applicable) <span style="float: right;">This information is continued on an additional sheet <input type="checkbox"/></span>	
In respect of those designations in which a European patent is sought, a sample of the deposited microorganism will be made available until the publication of the mention of the grant of the European patent or until the date on which the application has been refused or withdrawn or is deemed to be withdrawn, only by the issue of such a sample to an expert nominated by the person requesting the sample. (Rule 28(4) EPC)	
<b>D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE</b> (if the indications are not for all designated States)	
<b>E. SEPARATE FURNISHING OF INDICATIONS</b> (leave blank if not applicable)	
The indications listed below will be submitted to the International Bureau later (specify the general nature of the indications e.g., "Accession Number of Deposit")	

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## INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

(PCT Rule 13bis)

<b>A.</b> The indications made below relate to the microorganism referred to in the description on page <u>8</u> , line s <u>6 - 12</u>	
<b>B. IDENTIFICATION OF DEPOSIT</b> <span style="float: right;">Further deposits are identified on an additional sheet <input type="checkbox"/></span>	
Name of depositary institution AMERICAN TYPE CULTURE COLLECTION	
Address of depositary institution (including postal code and country) 12301 Parklawn Drive Rockville, Maryland 20852 US	
Date of deposit 26 September 1996	Accession Number 74392
<b>C. ADDITIONAL INDICATIONS</b> (leave blank if not applicable) <span style="float: right;">This information is continued on an additional sheet <input type="checkbox"/></span>	
In respect of those designations in which a European patent is sought, a sample of the deposited microorganism will be made available until the publication of the mention of the grant of the European patent or until the date on which the application has been refused or withdrawn or is deemed to be withdrawn, only by the issue of such a sample to an expert nominated by the person requesting the sample. (Rule 28(4) EPC)	
<b>D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE</b> (if the indications are not for all designated States)	
<b>E. SEPARATE FURNISHING OF INDICATIONS</b> (leave blank if not applicable)	
The indications listed below will be submitted to the International Bureau later (specify the general nature of the indications e.g., "Accession Number of Deposit")	

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## WHAT IS CLAIMED IS:

1. A method for the production of 1,3-propanediol from a recombinant organism comprising:

(i) transforming a suitable host organism with a transformation cassette comprising at least one of

(a) a gene encoding a glycerol-3-phosphate dehydrogenase activity;

(b) a gene encoding a glycerol-3-phosphatase activity;

(c) genes encoding a dehydratase activity;

(d) a gene encoding 1,3-propanediol oxidoreductase activity, provided that if the transformation cassette comprises less than all the genes of (a)-(d), then the suitable host organism comprises endogenous genes whereby the resulting transformed host organism comprises at least one of each of genes (a)-(d);

(ii) culturing the transformed host organism under suitable conditions in the presence of at least one carbon source selected from the group consisting of monosaccharides, oligosaccharides, polysaccharides, or a one-carbon substrate whereby 1,3-propanediol is produced; and

(iii) recovering the 1,3-propanediol.

2. The method of Claim 1 wherein the transformation cassette comprises all of the genes (a)-(d).

3. The method of Claim 1 wherein the suitable host organism is selected from the group consisting of bacteria, yeast, and filamentous fungi.

4. The method of Claim 3 wherein the suitable host organism is selected from the group of genera consisting of *Citrobacter*, *Enterobacter*, *Clostridium*, *Klebsiella*, *Aerobacter*, *Lactobacillus*, *Aspergillus*, *Saccharomyces*, *Schizosaccharomyces*, *Zygosaccharomyces*, *Pichia*, *Kluyveromyces*, *Candida*, *Hansenula*, *Debaryomyces*, *Mucor*, *Torulopsis*, *Methylobacter*, *Escherichia*, *Salmonella*, *Bacillus*, *Streptomyces* and *Pseudomonas*.

5. The method of Claim 4 wherein the suitable host organism is selected from the group consisting of *E. coli*, *Klebsiella spp.*, and *Saccharomyces spp.*

6. The method of Claim 1 wherein the transformed host organism is a *Saccharomyces spp.* transformed with a transformation cassette comprising the genes *dhaB1*, *dhaB2*, *dhaB3*, and *dhaT*, wherein the genes are stably integrated into the *Saccharomyces spp.* genome.

7. The method of Claim 1 wherein the transformed host organism is a *Klebsiella spp.* transformed with a transformation cassette comprising the genes GPD1 and GPD2.

8. The method of Claim 1 wherein the carbon source is glucose.

5 9. The method of Claim 1 wherein the gene encoding a glycerol-3-phosphate dehydrogenase enzyme is selected from the group consisting of genes corresponding to amino acid sequences given in SEQ ID NO:11, in SEQ ID NO:12, and in SEQ ID NO:13, the amino acid sequences encompassing amino acid substitutions, deletions or additions that do not alter the function of the  
10 glycerol-3-phosphate dehydrogenase enzyme.

10 10. The method of Claim 1 wherein the gene encoding a glycerol-3-phosphatase enzyme is selected from the group consisting of genes corresponding to amino acid sequences given in SEQ ID NO:33 and in SEQ ID NO:17, the amino acid sequences encompassing amino acid substitutions,  
15 deletions or additions that do not alter the function of the glycerol-3-phosphatase enzyme.

20 11. The method of Claim 1 wherein the gene encoding a glycerol kinase enzyme corresponds to an amino acid sequence given in SEQ ID NO:18, the amino acid sequence encompassing amino acid substitutions, deletions or additions that do not alter the function of the glycerol kinase enzyme.

25 12. The method of Claim 1 wherein the genes encoding a dehydratase enzyme comprise dhaB1, dhaB2 and dhbB3, the genes corresponding respectively to amino acid sequences given in SEQ ID NO:34, SEQ ID NO:35, and SEQ ID NO:36, the amino acid sequences encompassing amino acid substitutions, deletions or additions that do not alter the function of the dehydratase enzyme.

30 13. The method of Claim 1 wherein the gene encoding a 1,3-propanediol oxidoreductase enzyme corresponds to an amino acid sequence given in SEQ ID NO:37, the amino acid sequence encompassing amino acid substitutions, deletions or additions that do not alter the function of the 1,3-propanediol oxidoreductase enzyme.

14. A transformed host cell comprising:

(a) a group of genes comprising

- 35 (1) a gene encoding a glycerol-3-phosphate dehydrogenase enzyme corresponding to the amino acid sequence given in SEQ ID NO:11;  
(2) a gene encoding a glycerol-3-phosphatase enzyme corresponding to the amino acid sequence given in SEQ ID NO:17;  
(3) a gene encoding the a subunit of the glycerol dehydratase enzyme corresponding to the amino acid sequence given in SEQ ID NO:34;

(4) a gene encoding the  $\beta$  subunit of the glycerol dehydratase enzyme corresponding to the amino acid sequence given in SEQ ID NO:35;

(5) a gene encoding the  $\gamma$  subunit of the glycerol dehydratase enzyme corresponding to the amino acid sequence given in SEQ ID NO:36; and

5 (6) a gene encoding the 1,3-propanediol oxidoreductase enzyme corresponding to the amino acid sequence given in SEQ ID NO:37, the respective amino acid sequences of (a)(1)-(6) encompassing amino acid substitutions, deletions, or additions that do not alter the function of the enzymes of genes (1)-(6), and

10 (b) a host cell transformed with the group of genes of (a), whereby the transformed host cell produces 1,3-propanediol on at least one substrate selected from the group consisting of monosaccharides, oligosaccharides, and polysaccharides or from a one-carbon substrate.

# INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 97/20292

## A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 C12N15/53 C12N15/55 C12N15/60 C12P7/18 C12N9/04  
C12N9/16 C12N9/88

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C12P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P, X, L	WO 96 35796 A (DU PONT ; GENENCOR INT (US); LAFFEND LISA ANNE (US); NAGARAJAN VASA) 14 November 1996 see the whole document see abstract see claims 1-33 --- -/--	1, 3-6, 8, 12, 13



Further documents are listed in the continuation of box C.



Patent family members are listed in annex.

### \* Special categories of cited documents:

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
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- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

- "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- "&" document member of the same patent family

Date of the actual completion of the international search

10 March 1998

Date of mailing of the international search report

24/03/1998

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Lejeune, R

## INTERNATIONAL SEARCH REPORT

Int. l. Application No

PCT/US 97/20292

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	I-TEH TONG ET AL: "ENHANCEMENT OF 1,3-PROPANEDIOL PRODUCTION BY COFERMENTATION IN ESCHERICHIA COLI EXPRESSING KLEBSIELLA PNEUMONIAE DHA REGULON GENES" APPLIED BIOCHEMISTRY AND BIOTECHNOLOGY, vol. 34/35, 1992, pages 149-159, XP000578884 cited in the application see abstract see page 150, line 24 - line 29 see page 152; table 1 ----	1,3-6,8, 12,13
A	DE 37 34 764 A (HUELS CHEMISCHE WERKE AG) 3 May 1989 cited in the application see abstract see column 3; claim 1 ----	1
A	DANIEL R ET AL: "PURIFICATION OF 1,3-PROPANEDIOL DEHYDROGENASE FROM CITROBACTER FREUNDII AND CLONING, SEQUENCING, AND OVEREXPRESSION OF THE CORRESPONDING GENE IN ESCHERICHIA COLI" JOURNAL OF BACTERIOLOGY, vol. 177, no. 8, 1 April 1995, pages 2151-2156, XP000579775 cited in the application see abstract ----	13
A	TOBIMATSU T. ET AL.: "Cloning, sequencing and high level expression of the genes encoding adenosylcobalamin-dependent glycerol dehydrase of Klebsiella pneumoniae." JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 271, no. 37, 13 September 1996, pages 22352-22357, XP002057923 see abstract see page 22355; figure 5 see page 22356, column 1, line 2 - line 5 -----	12

# INTERNATIONAL SEARCH REPORT

Information on patent family members

Int. l. Application No

PCT/US 97/20292

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9635796 A	14-11-96	US 5686276 A AU 5678996 A EP 0826057 A	11-11-97 29-11-96 04-03-98
DE 3734764 A	03-05-89	NONE	